Final Annual Report

Structure-Function Studies on Glutathione S-Transferases (GSTs) from the Thai Malaria Vector *Anopheles dirus*. TRF BRG/08/2545

Associate Professor Albert J. Ketterman Ph.D. et al.

August 2004

TRF BRG/08/2545

Final Annual Report

Structure-Function Studies on Glutathione S-Transferases (GSTs) from the Thai Malaria Vector *Anopheles dirus*.

1. Assoc. Prof. Albert J. Ketterman	Institute of Molecular Biology and Genetics (IMBG), Mahidol University, Salaya Campus
2. Jantana Wongsantichon	IMBG, Mahidol University
3. Jeerang Wongtrakul	IMBG, Mahidol University
4. Rungrutai Udomsinprasert	IMBG, Mahidol University
5. Pakorn Winayanuwattikun	IMBG, Mahidol University
6. Ardcharaporn Vararattanavech	IMBG, Mahidol University
7. Juthamart Piromjitpong	IMBG, Mahidol University
8. Mashamon Mitprasat	IMBG, Mahidol University

Funded by the Thailand Research Fund (TRF)

Acknowledgements

The progress achieved for the last three years on this project was possible because of the efforts and contributions from the following people:

Institute of Molecular Biology and Genetics, Mahidol University

Technical help:
Jantana Wongsantichon

Ph.D. Students:
Jeerang Wongtrakul (funded by the Royal Golden Jubilee, now completed), Rungrutai Udomsinprasert (funded by the Royal Golden Jubilee), Pakorn Winayanuwattikun (funded by the Royal Golden Jubilee), Ardcharaporn Vararattanavech (funded by the Royal Golden Jubilee), Jantana Wongsantichon (funded by the Royal Golden Jubilee)

M.Sc. Students:
Thasaneeya Harnnoi (now completed), Satjana Pattanasak (now completed), Ardcharaporn Vararattanavech (now completed), Juthamart Piromjitpong, Mashamon Mitprasat

B.Sc. Student:
Katila Varivarn

Chiang Mai University
Dr. L. Prapanthadara
Dr. Jeerang Wongtrakul

Maejo University
Dr. Saengtong Pongjareankit

University of Western Australia, Australia
Dr. M.A. Bogoyevitch
Dr. M.C.J. Wilce

Australian National University
Dr. A.J. Oakley

Abstracts

The aim of this project is to characterize structure function relationships for the amino acids that can affect substrate specificity as well as ligand interactions in glutathione S-transferases (GST) from the Thai malaria vector *Anopheles dirus*. This Final Annual Report consists mainly of seven manuscripts that have been accepted for publication. The format is of separate studies, one in each manuscript. These studies involve characterization of structural amino acid residues that also appear to modulate enzymatic properties such as substrate and inhibitor specificity. Each study utilizes one of the enzymes and its crystal structure obtained from the research previously funded by the TRF. These seven manuscripts therefore describe on-going structure function studies.

บทคัดย่อ

งานวิจัยนี้มุ่งศึกษาความสำคัญของกรดอะมิโนที่มีต่อหน้าที่และโครงสร้างของเอนไซม์กลูตาไธโอน เอส - ทรานสเฟอเรส ในยุงพาหะนำโรคมาลาเรีย *Anopheles dirus* โดยศึกษาผลกระทบต่อความจำเพาะเจาะจงกับสารตั้งต้น รวมทั้งการจับกับสารอื่นๆ รายงานประจำปีฉบับนี้ได้แยกเป็นส่วนๆ ตามการศึกษาวิจัย ประกอบกับผลงานตีพิมพ์ทั้ง-สิ้น 7 บทความที่ได้รับการตอบรับตีพิมพ์แล้ว การวิจัยนี้ได้จำแนกกรดอะมิโนที่มีผลต่อทั้งโครงสร้างและคุณสมบัติของเอนไซม์ แต่ละการศึกษาใช้เอนไซม์และโครงร่างผลึกของเอนไซม์ที่ได้จากงานวิจัยก่อนหน้านี้ ซึ่งได้รับการสนับสนุนจาก สกว. บทความตีพิมพ์ทั้ง 7 บทความจึงเป็นความก้าวหน้าของการศึกษาดังกล่าว

Executive Summary

This Final Annual Report consists mainly of seven manuscripts that have been accepted for publication. The format is of separate studies, one in each manuscript.

Glutathione S-transferases (GSTs) are dimeric proteins that play a major role in cellular detoxification. The GSTs in mosquito *Anopheles dirus* species B, an important malaria vector in South East Asia, are of interest because they can play an important role in insecticide resistance. In this study, we characterize the AdGST D3-3 that is an alternatively spliced product of the *adgst1*AS1 gene. The data from the crystal structure of GST D3-3 shows that Ile-52, Glu-64, Ser-65, Arg-66 and Met-101 interact directly with glutathione. To study the active site function of these residues, alanine substitution site-directed mutagenesis was performed resulting in five mutants; I52A, E64A, S65A, R66A and M101A. Interestingly, E64A expresses in *E.coli* in inclusion form suggesting that this residue is involved with the tertiary structure or folding property of this enzyme. However, I52A, S65A, R66A and M101A mutants were purified by glutathione affinity chromatography and the enzyme activity characterized. On the basis of steady state kinetics, difference spectroscopy, unfolding and refolding studies, it is concluded that these residues: (1) contribute to the affinity of the G-site for GSH; (2) influence GSH thiol ionization; (3) participate in kcat regulation by affecting the rate-limiting step of the reaction; and in the case of Ile-52 and Arg-66 influenced structural integrity and/or folding of the enzyme. The structural perturbations from these mutants are probably transmitted to the H-site through changes in active site topology or through effects on GSH orientation. Therefore these active site residues appear to contribute to various steps in the catalytic mechanism as well as an influence on the packing of the protein.

In mammalian systems, detoxication enzymes of the glutathione S-transferase family (E.C. 2.5.1.18; GST) regulate Jun-N-terminal kinase (JNK) signal transduction by interaction with JNK itself or other proteins upstream in the JNK pathway. Here we have studied GSTs and their interaction with components of the JNK pathway from *Diptera*. We have evaluated the effects of four delta class *Anopheles dirus* GSTs, GSTD1-1, D2-2, D3-3 and D4-4, on the activity of full-length recombinant *Drosophila* HEP (MAPKK7) and the *Drosophila* JNK as well as the reciprocal effect of these kinases on GST activity. Interestingly, even though these four GSTs are alternatively-spliced products of the same gene and share > 60% identity, they exerted different effects on JNK activity. GSTD1-1 inhibited JNK activity whereas the other three GST isoforms activated JNK. GSTs D2-2, D3-3 and D4-4 were inhibited between 50-80% by HEP or JNK but GSTD1-1 was not inhibited by JNK. However, there were some similarities in the actions of HEP and JNK on these GSTs. For example, binding constants for HEP or JNK inhibiting a GST were similar (20-70 nM). Furthermore, upon incubation of the GSTs with JNK both JNK and the GSTs changed catalytic properties. The substrate specificities of both GSTs and JNK were also altered upon their co-incubation. In addition, glutathione modulated the effects of JNK on GST activity. These data emphasize that different GST spliceforms possess different properties, both in their catalytic function and in their regulation of signaling via the JNK pathway.

A variant form of an *Anopheles dirus* glutathione S-transferase (GST), designated AdGSTD4-4, possesses a single amino acid change of leucine to arginine (Leu-103-Arg). Although residue 103 is outside of the active site, it has major effects on enzymic properties. To investigate these structural effects, site directed mutagenesis was used to generate mutants by changing the non-polar leucine to alanine, glutamate, isoleucine, methionine, asparagine, or tyrosine. All of the recombinant GSTs showed approximately the same expression level at 25°C. Several of the mutants lacked glutathione (GSH)-binding affinity but were purified by S-hexyl-GSH-based affinity chromatography. However the protein yields (70-fold lower), as well as the GST activity (100-fold lower), of Leu-103-Tyr and Leu-103-Arg purifications were surprisingly low and precluded the performance of kinetic experiments. Size-exclusion chromatography showed that both GSTs Leu-103-Tyr and Leu-103-Arg formed dimers. Using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH substrates to determine kinetic constants it was demonstrated that the other Leu-103 mutants possessed a greater K_m towards GSH and a differing K_m towards CDNB. The V_{max} ranged from 44.7 to 87.0 μmol/min per mg (wild-type, 44.7 μmol/min per mg). Substrate-specificity studies showed different selectivity properties for each mutant. The structural residue Leu-103 affects the active site through H-bond and van-der-Waal contacts with six active-site residues in the GSH binding site. Changes in this interior core residue appear to disrupt internal packing, which affects active-site residues as well as residues at the subunit–subunit interface. Finally, the data suggest that Leu-103 is noteworthy as a sensitive residue in the GST structure that modulates enzyme activity as well as stability.

The Cys69 residue of an *Anopheles dirus* glutathione S-transferase isoform (AdGSTD3-3) was characterized to elucidate its contribution in both catalysis and structural support. Nine mutants were generated at this position by replacing the residue with polar, non-polar and charged residues. The polar residues changed the V_m of the enzymes. With non-polar residues, the enzymes were unable to fold and were expressed in the insoluble inclusion form. With charged residues, the soluble enzyme yields were only 3% of the wild type protein. Molecular dynamics simulation also was performed to understand the changes in the enzyme structure. These findings are additional evidence of the importance of structural residues that affect the enzymatic properties such as V_m, K_m and enzyme specificity.

To elucidate how non-active site residues support the catalytic function, five selected residues of AdGSTD3-3 isoenzyme were changed to AdGSTD1-1 residues by means of site-directed mutagenesis. Analysis of the kinetic parameters indicated that Cys69Gln and Asp150Ser showed marked differences in V_{max} and K_m compared with the wild type enzyme. Both residues were characterized further by replacement with several amino acids. Both the Cys69 and Asp150 mutants showed differences with several GST substrates and inhibitors including affecting the interactions with pyrethroid insecticides. Cys69 and Asp150 mutants possessed a decreased half-life relative to the wild type enzyme. The Asp150 mutation appears to affect neighboring residues that support two important structural motifs, the N-capping box and the hydrophobic staple motif. The Cys69 mutants appeared to have subtle conformational changes near the active site residues resulting in different conformations and also directly affecting the active site region. The results show the importance of the cumulative effects of residues remote from the active site and demonstrate that minute changes in tertiary structure play a role in modulating enzyme activity.

This study was designed to characterize residues in the glutathione binding site of AdGSTD4-4 from the mosquito malaria vector *Anopheles dirus*. The data revealed that Leu33, His38 and His50 each play a role in enzyme catalysis and glutathione binding. The mutants of these three residues also displayed differences in hydrophobic substrate specificity, suggesting that changes in the active site conformation occurred. Differences in conformations was also suggested by protein stability changes. These results indicate that residues in the glutathione binding site are not only important in the catalytic function but also play a role in the structural integrity of the enzyme.

A new *Anopheles dirus* glutathione S-transferase (GST) has been obtained and named adGST4-1. Both genomic DNA and cDNA for heterologous expression were acquired. The genomic sequence was 3188 bp and consisted of the GST gene as well as flanking sequence. The flanking sequence was analyzed for possible regulatory elements that would control gene expression. In *Drosophila* several of these elements have been shown to be involved in development and cell differentiation. The deduced amino acid sequence has low identity compared with the four alternatively spliced enzymes, adGST1-1 to 1-4, from another *An. dirus* GST gene *adgst1AS1*. The percent identities are 30–40% and 11–12% comparing adGST4-1 to insect GSTs from Delta and Sigma classes, respectively. Enzyme characterization of adGST4-1 shows it to be distinct from the other *An. dirus* GSTs because of low enzyme activity for customary GST substrates including 1-chloro-2, 4-dinitrobenzene (CDNB). However, this enzyme has a greater affinity of interaction with pyrethroids compared to the other *An. dirus* GSTs.

Keywords: Glutathione transferase; *Anopheles dirus*; Mosquito; Mutagenesis; Structure–function study; protein tertiary structure.

Objectives

The aim of this project is to characterize structure function relationships for the amino acids that can affect substrate specificity as well as ligand interactions in glutathione S-transferases (GST) from the Thai malaria vector *Anopheles dirus*. To achieve this goal the project entails continuing several aspects of the research previously funded by the Thailand Research Fund. These aspects include the following:

1. To continue to obtain genomic DNA from our *An. dirus* genomic library that contains GST coding sequence. This will yield new recombinant GST enzymes to characterize.
2. To continue to express acquired GST enzymes for kinetic characterization studies including enzyme sequence variants generated by site-directed mutagenesis.
3. To continue to obtain more GST crystal structures and to use the now available *An. dirus* GST crystal structures to provide a basis for understanding the mechanism of GSTs in their role in resistance to insecticides.

This Final Annual Report consists mainly of seven manuscripts that have been accepted for publication. The format is of separate studies, one in each manuscript. These studies involve characterization of structural amino acid residues that also appear to modulate enzymatic properties such as substrate and inhibitor specificity. Each study utilizes one of the enzymes and its crystal structure obtained from the research previously funded by the TRF. These seven manuscripts therefore describe on-going structure function studies.

Reciprocal Regulation of Glutathione *S*-Transferase Spliceforms and the *Drosophila* c-Jun N-terminal Kinase Pathway Components

Rungrutai Udomsinprasert*, Marie A. Bogoyevitch¶ and Albert J. Ketterman*

**Institute of Molecular Biology and Genetics,*
Mahidol University, Salaya Campus, Nakorn Pathom 73170, Thailand

¶Cell Signalling Laboratory, Biochemistry and Molecular Biology,
University of Western Australia, 35 *Stirling Highway, Crawley, WA* 6009, *Australia*

Corresponding author. Fax: +66-2-441-9906.
A.J. Ketterman
E-mail address: frakt@mahidol.ac.th

Running title: GST spliceform-specific JNK regulation

SUMMARY (243 words)

In mammalian systems, detoxication enzymes of the glutathione S-transferase family (E.C. 2.5.1.18; GST) regulate Jun-N-terminal kinase (JNK) signal transduction by interaction with JNK itself or other proteins upstream in the JNK pathway. Here we have studied GSTs and their interaction with components of the JNK pathway from *Diptera*. We have evaluated the effects of four delta class *Anopheles dirus* GSTs, GSTD1-1, D2-2, D3-3 and D4-4, on the activity of full-length recombinant *Drosophila* HEP (MAPKK7) and the *Drosophila* JNK as well as the reciprocal effect of these kinases on GST activity. Interestingly, even though these four GSTs are alternatively-spliced products of the same gene and share > 60% identity, they exerted different effects on JNK activity. GSTD1-1 inhibited JNK activity whereas the other three GST isoforms activated JNK. GSTs D2-2, D3-3 and D4-4 were inhibited between 50-80% by HEP or JNK but GSTD1-1 was not inhibited by JNK. However, there were some similarities in the actions of HEP and JNK on these GSTs. For example, binding constants for HEP or JNK inhibiting a GST were similar (20-70 nM). Furthermore, upon incubation of the GSTs with JNK both JNK and the GSTs changed catalytic properties. The substrate specificities of both GSTs and JNK were also altered upon their co-incubation. In addition, glutathione modulated the effects of JNK on GST activity. These data emphasize that different GST spliceforms possess different properties, both in their catalytic function and in their regulation of signaling via the JNK pathway.

INTRODUCTION

Glutathione S-transferases (EC 2.5.1.18; GSTs) are a superfamily of multifunctional enzymes involved in normal cellular metabolism as well as the detoxication of various hydrophobic endogenous and xenobiotic compounds [1]. The central importance of GSTs in detoxication lies in their unique capacity to conjugate glutathione with a wide variety of compounds [2]. This detoxification reaction is of critical importance in cell survival. As a consequence, GSTs have been found virtually in all organisms and are currently grouped into at least 10 classes based on their primary sequence similarity, substrate specificity, immunological properties, tertiary structure and quaternary structure [3]. Changes in glutathione levels have also been associated with the activation of a stress response [4] and GSTs can protect against electrophiles and oxidative stress by altering cellular glutathione levels [1]. GSTs also have glutathione peroxidase activity under conditions of oxidative stress and they have been further implicated in a range of physiological roles such as signal transduction, cell proliferation, differentiation and apoptosis [5].

Various GST classes have been shown to be involved in these broader physiological roles. For example, GST omega protected cells from apoptosis induced by Ca^{2+} mobilization from intracellular stores via its ability to regulate the Ca^{2+} channel activity of the Ryanodine receptor [6]. Because the Bcl-2 family member Bax regulates programmed cell death by promoting apoptosis [7], a role for GST theta in regulation of apoptotic cell death has also been suggested following its identification as a Bax-interacting protein [8].

Other interactions of GSTs to alter intracellular signal transduction events have been reported. Most often these implicate GST in the regulation of the Jun N-terminal kinase (JNK) signal transduction pathway. JNK is a member of the MAPK family which is conserved across all eukaryotes ranging from yeast and insects to mammals [9]. JNK has been implicated in a variety of biological functions in response to stress, and the transmission of signals via the JNK pathway is achieved by sequential phosphorylation and activation of the pathway kinase components, the MAPKKKs, the MAPKKs and the MAPKs, respectively. The activation of JNK relays extracellular cues to transcription factors such as c-Jun [10], ATF2 [11] and Elk1 [12], thereby regulating gene expression, cellular homeostasis, differentiation, apoptosis and cell death. Not only is the induction of c-*jun* and c-*fos* transcription dependent on JNK, but Jun and Fos can induce the transcription of

xenobiotic-metabolizing enzymes such as GST [13]. Thus, c-Jun is directly involved in GST pi expression *in vivo* [14]. This suggests a role of JNK in the induction of a cellular defense program against cytotoxic xenobiotics.

JNK pathway components and GSTs are evolutionally conserved across mammals and insects. Different mammalian GST classes such as GST pi and GST mu have been reported to interact with different stress kinase proteins in the JNK pathway. For example, GST pi is a JNK regulatory protein, and its association with JNK maintains a low basal level of JNK activity in the non-stressed cell [15]. The lack of GST pi increased constitutive JNK activity *in vivo* and therefore regulated the expression of genes that were specific downstream targets of the JNK pathway [16]. Moreover, GST pi coordinates ERK/p38/IKK activation as part of the mechanism underlying its ability to elicit protection against H_2O_2-induced cell death [17]. In contrast, GST mu interacts with Apoptosis Signal-regulating Kinase 1 (ASK1), an upstream activating kinase of JNK that participates in cell death [18].

Here we evaluate the interaction of GST and kinase proteins in a *Dipteran* system using four different spliceforms of *Anopheles dirus* delta class GSTs and two different *Drosophila* kinase proteins, *Drosophila* HEP7 (HEP) and *Drosophila* JNK (JNK). The *Drosophila* JNK pathway viewed as a linear cascade [19], comprises the *Hemipterous* (HEP or DMKK7) [20], *basket* (JNK) [21] and *D-Jun* [22], which are homologous proteins to mammalian MAPKK7, JNK and c-Jun, respectively. The four *Anopheles* GSTs used in this study are alternatively spliced products from a single gene [23]. To elucidate the mechanism by which GSTs modulate the JNK signaling pathway, we assessed both GST and kinase activities to provide evidence for direct protein-protein interactions and to measure binding affinity. Our results show that the GSTs interact with protein kinases, that the different GST isoforms appear to possess different regulatory mechanisms in the JNK pathway, and that JNK interaction also affects GST activities. This is the first report of the reciprocal regulation of GST and JNK pathway activities.

EXPERIMENTAL PROCEDURES

Preparation of DNA Constructs. The alternatively spliced products, GSTD1-1, D2-2, D3-3 and D4-4, were cloned into a pET3a vector [24]. The recombinant proteins in the *Drosophila* JNK pathway consisting of *Drosophila* HEP7 (HEP; Genbank accession number AAB63449.1), *Drosophila* JNK (JNK and also known as *basket*; Genbank accession number AAB97094.1) and the transactivation domain of *Drosophila* Jun (amino acids 1-104; Jun 1-104; Genbank accession number P18289) were obtained by RT-PCR from adult *Drosophila melanogaster*. The PCR products were then cloned into a pET28b vector (Stratagene). The HEP recombinant plasmid was also used as the template for site-directed mutagenesis method to construct a constitutively-active HEP mutant (HEP_{3E}). The mammalian MKK7-β1 isoform mutant ($MKK7_{3E}$) has constitutive kinase activity following the substitutions of Ser^{271}, Thr^{275} and Ser^{277} to Glu [25], we therefore altered the three homologous residues (Ser^{348}, Thr^{352} and Ser^{354}) of *Drosophila* HEP7 to Glu by two step PCR using a Quik Change™ Site-Directed Mutagenesis kit (Stratagene). All recombinant clones were identified by restriction digest of the plasmids and confirmed by full-length sequencing in both directions using a BigDye™ Terminator Cycle Sequencing Kit (Perkin–Elmer).

Preparation of Recombinant Proteins. The GST proteins were expressed and purified using either GSTrap or S-hexyl-glutathione affinity chromatography [24]. The four recombinant proteins HEP, HEP_{3E}, JNK and Jun 1-104 were expressed as histidine fusion proteins. The JNK and Jun 1-104 recombinant proteins were expressed as soluble proteins and purified using a standard Ni^{2+}-NTA column protocol (Amersham Pharmacia Biotech). In contrast, HEP and HEP_{3E} recombinant proteins were expressed mainly in inclusion bodies. Therefore, these HEP and HEP_{3E} were purified using Ni^{2+}-NTA column chromatography under denaturing conditions and renatured by slow dialysis using the Roti®-Fold reagent (Carl Roth GmbH+ Co.). Protein concentrations were determined using the BioRad protein reagent with serum bovine albumin as the standard [26].

GST Activity Assays. GST activity was measured by the conjugation of glutathione (GSH) with the hydrophobic substrates 1-chloro-2,4-dinitrobenzene (CDNB; Aldrich), 1,2-dichloro-4-nitrobenzene (DCNB; Fluka), *p*-nitrobenzyl chloride (PNBC; Aldrich) and *p*-nitrophenyl bromide (PNPB; Aldrich) [24]. We used the CDNB conjugation as our standard assay because GST specific activity for this substrate was highest [24].

Effects of Protein Kinases HEP and JNK on GST activity. The effects of HEP and JNK on GST activity were examined by incubating GSTs and kinase proteins in a 1:1 molar ratio at room temperature (27-30°C) for 5 min. The GST activity was measured in the presence and absence of kinase proteins. The effects on GST activity were evaluated by:

i) the % inhibition of GST activity. The % inhibition was determined by measuring GST activity in the presence of HEP or JNK as well as without kinase proteins as the control.

ii) the type of inhibition and affinity binding (K_i). GST and kinase protein interactions were performed by varying concentration of CDNB from 0.05 to 3.0 mM and measuring kinetic parameters for CDNB and GSH conjugation [24]. The kinetic parameters and K_i were determined by both linear and non-linear regression analysis using GraphPad Prism 2.01 software.

iii) the change of GST substrate specificity by JNK. GST and JNK were incubated in a 1:1 molar ratio and the effects of JNK on GST activity were determined using the hydrophobic substrates for GST, as mentioned above. A positive or negative change of GST activity towards a substrate, when compared to activity in the absence of JNK, indicated a substrate selectivity change of GST.

iv) the effect of glutathione (GSH) on GST activity in the presence of JNK. A 1:1 molar ratio of GST: JNK was incubated at room temperature for 5 minutes in the presence and absence of 2 mM GSH. The GST activity was determined towards its hydrophobic substrates as described above.

In vitro Protein Kinase Assays. Constitutively active HEP_{3E} was used to activate JNK, then both HEP_{3E} and Jun 1-104 were assessed as JNK substrates [21,22]. HEP_{3E}, JNK and Jun 1-104 in 1:2:10 molar ratio were incubated in 20 mM HEPES, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, pH 7.6, containing 500 μM dithiothreitol, 100 μM sodium orthovanadate and supplemented with 20 μM ATP, 3 μCi of [γ-^{32}P] ATP. The phosphorylation reactions were performed for 25 min at room temperature and separated by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography and quantitated by Cerenkov counting.

Effects of GST on Protein Kinase Activity. The recombinant GSTs were incubated with kinase proteins in a 10:1 molar ratio for 10 min at room temperature. Kinase activity was then measured as described above.

RESULTS

JNK and HEP can inhibit the activity of delta GST spliceforms.

Previous studies have shown that GSTs of the mammalian pi and mu classes are capable of modulating the JNK pathway through their inhibition of JNK and interaction with ASK, respectively [18]. In the present study we have evaluated whether GSTs of the *Dipteran* delta class could interact with two kinases from *Drosophila,* either JNK or its upstream kinase HEP, by measuring both the effects of the kinases on GST activity and the effects of the GSTs on kinase activity.

Previously a comprehensive study of six GST isoforms yielded estimates of total GST concentrations in eukaryotic cells, depending on tissue type, ranging from 0.1 to 1.4 nmol GST per mg of total soluble protein [27]. From our laboratory data we estimate *Drosophila* SL2 cells to contain 66 pg soluble protein/cell. Assuming a tissue culture cell volume of 1 pL [28], a total GST cell concentration of 7 to 90 μM can be estimated. A single MAPK protein has been estimated to have cellular concentrations of 1 to 3 μM [28]. In *Anopheles gambiae* it has been reported that there are a possible 32 different soluble GST encoding transcripts with 31 being found in the adult mosquito [29]. This is similar to the number of 37 putative GST genes identified in *Drosophila* [29]. It was also shown that depending on specific tissue/cell type a single GST isoform could vary between 0.15 to 45 μM [27]. However, these concentration determinations do not address the issue of compartmental localization known to be important in controlling message propagation down signal transduction pathways. We therefore chose an intermediate concentration range and used a 1:1 ratio for the GST:JNK experiments.

We began with an evaluation of the effects of JNK and HEP on the activity of four delta GST spliceforms. Alternative splicing is a major mechanism of generating protein diversity in higher eukaryotes [30]. These four GSTs possess 61-77% amino acid identity and are products of the *Adgst1AS1* gene [23,24]. They share an untranslated exon 1 and a translated exon 2 that codes for 45 amino acids at the N-terminus. These two exons are spliced to one of four alternative exons 3, namely 3A, 3B, 3C or 3D. This generates four

different mature transcripts coding for proteins of 209 to 219 amino acids that have been called D4-4, D3-3, D2-2, and D1-1, respectively. These spliceforms therefore differ in their C-terminal amino acids only.

As shown in Table 1, GST activities as assessed using the standard CDNB assay were decreased in the presence of a 1:1 molar concentration of HEP. The activities of the GST spliceforms D1-1 and D2-2 were the greatest affected, but even the inhibition of GST D3-3 and GST D4-4 was greater than 20%. A parallel series of experiments in which JNK was incubated with each delta GST spliceform also showed that JNK could inhibit the activity of GST D2-2, D3-3 and GST D4-4 towards CDNB. However, under these assay conditions, no inhibition of GST D1-1 activity could be observed (Table 1). The differences in the effects of the HEP and JNK on the activities of the GST spliceforms would appear to be the result of different interactions with the different amino acids in the C-terminus of each GST spliceform.

The mechanism of GST inhibition by HEP and JNK.

A more detailed kinetics study of inhibition was undertaken to yield data on the affinity of binding (K_i) and the mechanism of interaction for these four GST spliceforms and the kinases HEP and JNK (Table 2). With the exception of the JNK and GST D1-1 which showed no inhibition of GST activity, the inhibition of the GST spliceforms by JNK or by HEP was non-competitive with respect to its substrate CDNB. This indicated the interaction with each kinase did not block the GST active site despite inhibiting its transferase activity. This interaction was also of high affinity with estimates of K_i in the range of 20-70 nM for GST D1-1, GST D2-2 and GST D3-3 (Table 2). For GST D4-4, the K_i values were higher, being in the range of 100-200 nM, however all of these interactions were approximately three to four orders of magnitude greater affinity than the interactions of GSTs with glutathione [24]. Furthermore, this data shows that HEP interacts with all GST delta spliceforms tested. JNK also interacts with GST D2-2, D3-3 and D4-4, but it was not possible to observe an interaction of JNK with GST D1-1 in this experiment.

Modulation of Protein Kinase Activity in the Presence of GST delta spliceforms.

We next tested the reciprocal regulation of GST delta spliceforms on protein kinase activity. We began by assessing JNK activity towards its physiological substrate Jun. As shown in Figure 1A, GST D1-1 inhibited the ability of JNK to phosphorylate Jun by approximately

50%. In contrast the inclusion of GST D2-2, D3-3, or D4-4 increased JNK activity by up to 170%. Thus, despite JNK not inhibiting GST D1-1 activity towards CDNB (Table 1), GSTD1-1 was able to inhibit JNK activity towards Jun (Figure 1A).

We next repeated these protein kinase assays, but without the inclusion of the Jun substrate protein. In this way, we could assess the actions of the GST delta spliceforms on both HEP_{EE} and JNK activity using the ability of HEP_{EE} to phosphorylate JNK as well as the ability of JNK to phosphorylate HEP_{EE} [21]. As shown in Figure 1B, the incubation of JNK alone did not result in its significant autophosphorylation. Similarly prolonged incubation of HEP_{EE} alone did not result in its significant autophosphorylation (results not shown). The inclusion of HEP_{EE} with JNK resulted in weak phosphorylation of both HEP_{EE} and JNK proteins. When the GST delta spliceforms were also included, the most striking differences were noted with GST D2-2 and GST D3-3 which increased the phosphorylation of both HEP_{EE} and JNK proteins up to 6-fold. Furthermore, GSTD1-1 and D4-4 appeared to inhibit the phosphorylation of HEP_{EE} whilst not inhibiting the phosphorylation of JNK. These results therefore show that GSTs can affect the activities of both JNK towards HEP_{EE} and HEP_{EE} towards JNK. They also show that GSTs were not JNK substrates because no phosphorylated GST protein was observed even upon prolonged exposure of the autoradiographs. This is consistent with these GST proteins lacking a consensus phosphorylation site for MAPKs, namely S/TP or PXS/TP [31].

The JNK affected GST substrate specificity.

We next evaluated whether JNK changed the substrate specificity of the delta GST spliceforms. First, the specific activities of each delta GST spliceform were determined for the substrates CDNB, DCNB, PNBC and PNPB in the absence of any JNK protein. The results are presented in Table 3. The data illustrate the striking differences in enzymatic properties among these four GSTs. For example, 10-fold differences in specific activity are noted for the CDNB substrate. Likewise 8-fold differences for DCNB, 17-fold differences for PNBC substrate, and greater than 20-fold differences for PNPB are noted. These differences must arise from the differences in amino acids at the C-terminus. These C-terminal residues contribute to the H-site and have been shown to determine substrate specificity for different compounds within each class and amongst different classes [32-34].

The changes in GST substrate specificity upon incubation with JNK are shown in Figure 2. Some striking differences were observed. For example, GST D1-1 activity toward CDNB

was not influenced when incubated in the presence of JNK, nonetheless it was dramatically increased (approximately 2500-fold) towards PNPB. Likewise, incubation with JNK increased GST D2-2 activity using PNBC and PNPB whereas it was decreased using other substrates. Both D3-3 and D4-4 displayed an increase in activity towards PNPB, however the activities were decreased toward other substrates. These data suggest the JNK interaction provokes a set of different conformational changes in each GST, thus affecting the active site topologies, for instance changes in hydrophobicity and size through residue movement [35], in a dissimilar manner.

The effect of glutathione on GST and JNK interaction.

Under normal cellular conditions, intracellular GSH concentrations are thought to be in the range 1-10 mM. At these concentrations, GSH would usually be bound in the GST active site [36], and therefore we evaluated whether the presence of GSH would alter the effects of GSTs on JNK activities. In the presence of 2mM GSH, JNK had different effects on the delta GST spliceforms for the GST substrates, CDNB, DCNB, PNBC and PNPB (Figure 3). Specifically, when activities of the GST D1-1 and GST D2-2 spliceforms were assessed following preincubation in the absence (Figure 3A) and presence (Figure 3B) of glutathione, the most striking differences were noted for activities towards the CDNB substrate. GST D1-1 was now activated by JNK following preincubation in the presence of GSH and GST D2-2 was also activated rather than inhibited. We suggest the known changes of GSH binding that result in GST induced-fit conformational changes [37] could contribute to the changes noted in the GST-JNK interaction. These changes will be observed differently depending on the GST specific isoforms and substrate employed.

GSH also affected JNK activity by inducing JNK autophosphorylation (Figure 4A) whilst attenuating Jun phosphorylation (Figure 4B). As shown in the presence of Jun the autophosphorylation of JNK was decreased, however the GST and GSH induced-fit conformational change induced a 3.5-fold increase in JNK autophosphorylation. The data also identifies glutathione as a critical molecule directly involved in JNK regulation by controlling JNK substrate selectivity and specificity of interaction. Direct interaction of GSH and kinases has been reported previously for several isoforms of PKC that were shown to be inhibited by GSH [38]. High GSH concentrations have been shown to inhibit the sphingomyelin/ceramide cycle where generation of ceramide leads to cell cycle arrest and apoptosis [39]. In addition, the depletion of GSH in the cell during oxidative stress exerted

negative regulation over protein kinase C (PKC) isozymes [38] as well as JNK/p38 pathway [4]. GSH has been shown to be a potent inhibitor of JNK activation suggesting the existence of specific cellular components involved in JNK activation in response to different forms of cellular stress [4]. We suggest GSTs may be one of these components.

DISCUSSION

In the present study, we characterized the non-enzymatic function of GST in the regulation of stress-activated kinase proteins of the JNK pathway as well as the reciprocal regulation of GST by proteins of the JNK pathway. In insects, HEP or hemipterous is a homolog to mammalian MAPKK7 (MKK7) whereas the single *Drosophila* JNK or basket protein corresponds to the ten human JNK isoforms with a 61-75% amino acid identity [21]. Furthermore, GSTs in insects currently fall into 6 classes, with the delta class being the best-studied [29,40,41]. Here we have concentrated on the study of 4 spliceforms of a single delta class GST, GSTs D1-1, D2-2, D3-3 and D4-4. Interestingly, the splicing of these GSTs generates products that share the same N-terminal 45 amino acids of the conserved glutathione binding region [24]. Any differences in the actions in these spliceforms must therefore arise from the C-terminal regions of these proteins and we have previously shown that their substrate specificities, steady-state kinetics with respect to both GSH and CDNB, and inhibition kinetics to the pyrethroid insecticide permethrin are very different [24].

Our results in this present study have extended our previous studies by demonstrating an interaction between the *Dipteran* GSTs D1-1, D2-2, D3-3 and D4-4 and the JNK pathway components, JNK and HEP. Our inhibition study showed different interactions of these kinases with the GST splice products, particularly as seen with the inhibition of several of the GST spliceforms by JNK (Table 1). We observed, using the standard CDNB assay, that GSTD1-1 showed no inhibition whereas the remaining GSTs were inhibited by their preincubation with JNK protein. We suggest that the interaction with the kinase could change the GST conformation and this results in different GST enzymatic activity.

This is also the first report of the activation of JNK activity by their preincubation with GST. Previously, GSTs have been reported to serve as negative regulators in the JNK pathway, acting either on JNK or ASK1 [15,18]. Here we report that although some alternatively spliced GSTs inhibit JNK activity other spliceforms also may function as JNK activator proteins. This data again highlights the functional diversity of the GST spliceforms. Moreover, our study shows the GSTs can associate with the immediate JNK upstream

activator kinase in Drosophila, HEP. Hence, GST may play additional roles in regulation of kinase proteins in the JNK pathway through an, as yet, unknown mechanism.

As a consequence of the different effects of GSTD4-4 on JNK activity as shown by an increased phosphorylation of c-Jun (Figure 1A) and a decreased phosphorylation of HEP (Figure 1B), we suggest the GSTs such as D4-4 may contribute to a change of JNK substrate selectivity. Intriguingly, GST could be a pivotal molecule to switch the JNK downstream cascade direction by changing activation of transcriptional machinery components and thereby gene expression. One critical determinant of MAP kinase specificity and efficacy is the docking motif on the kinase surface which interacts with the substrate target site. A structural change of JNK upon interaction with GST may impact on this region critical for specificity determination [42,43]. Nonetheless, JNK has many functions in controlling cell stress responses, and it is possible that the GST-JNK interaction changes substrate specificities of JNK for other JNK substrates such as ATF2 [11], Elk-1[12] or p53 [44]. GST and JNK interaction would therefore function as switches or modulators for the various JNK processes upon stimulation by cellular stresses (Figure 5). Due to the nature of the signaling process, there must be other molecules participating in regulation of the GST - JNK interaction. Since there are various classes and isoforms of GSTs [1,2] and JNKs [9] that are widely distributed in different tissues, the signaling specificity of GST and JNK interactions may be also controlled by the specific classes and isoforms present in any particular cell type. Previously it was shown that distinct classes of GSTs played particular roles by interacting with different kinase proteins [15,18]. Here we report that different isoforms of the same class of GST interact with JNK and modulate JNK activity in different ways. The variety of GST isoforms may be one of the keys determining signal specificity and controlling a particular cell's biological response.

The GST and JNK interaction may occur through the C-terminus of JNK which has been reported as being important for providing direct protein-protein interaction with GSTP1-1 [45]. JNK has also been shown to interact electrostatically with other signaling molecules via a conserved docking motif called the CD (common docking) domain [46]. The conserved polar residues within the CD domain may serve as energetic hot spots which increase the specific protein-protein interaction [47,48]. In addition, hydrogen bonding and molecular surface shape complementarity are vital criteria as a basis for protein docking [49]. Thus the variation in surface residues of a GST interacting with a particular JNK may induce distinct conformational changes yielding functional changes in both proteins. A shift in substrate specificity arising from this association of GST and JNK, in addition to the

presence of different GST isoforms generated in the course of natural molecular evolution [50], would be a useful feature for a detoxication mechanism already well known for recognizing diverse substrate compounds.

In summary, the data show that distinct isoforms of GSTs specifically interact with JNK with different effects. Intriguingly, even though the studied GST isoforms are alternatively spliced products sharing greater than 60% amino acid identity, the GST proteins displayed contrary roles in regulation of JNK. It suggests the specific isoforms of GSTs present may be important in controlling the final cellular response. The present study provides new insight into the mechanism of GSTs in regulating and conferring specificity of stress kinase proteins in the JNK pathway.

FOOTNOTES

Abbreviations:

JNK; c-Jun N-terminal kinase, MAPKK; Mitogen activated protein kinase kinase, MAPKKK; MAP kinase kinase kinase, HEP; Hemipterous, ASK1; Apoptosis signal regulating kinase 1, PKC; Protein kinase C, ATF2; activating transcription factor 2, GST; Glutathione S-Transferase, GSH; Glutathione, CDNB; 1-chloro-2,4-dinitrobenzene, DCNB; 1,2-dichloro-4-nitrobenzene, PNBC; *p*-nitrobenzyl chloride, PNPB; *p*-nitrophenyl bromide.

Acknowledgements:

We are particularly grateful to Assoc. Prof. Dr. Warawut Chulalakasananukul, Genetics Program, Department of Botany, Chulalongkorn University, Bangkok Thailand for donating the *Drosophila melanogaster* adults used in cloning of the *Drosophila* HEP, JNK and Jun. This work was supported by the Thailand Research Fund (TRF), a Royal Golden Jubilee Scholarship (R.U.) and Royal Golden Jubilee Grant (R.U.).

Reference List

1 Hayes, J. D. and Pulford, D. J. (1995) The glutathione S-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. CRC Crit. Rev. Biochem. Molec. Biol. **30**, 445-600

2 Armstrong, R. N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem. Res. Toxicol. **10**, 2-18

3 Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem. J. **360**, 1-16

4 Wilhelm, D., Bender, K., Knebel, A., and Angel, P. (1997) The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. Mol. Cell. Biol. **17**, 4792-4800

5 Ketterer, B. (2001) A bird's eye view of the glutathione transferase field. Chemico-Biological Interactions **138**, 27-42

6 Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G., and Board, P. (2001) The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. J. Biol. Chem. **276**, 3319-3323

7 Fesik, S. W. (2001) Insights into programmed cell death through structural biology. Cell **103**, 273-282

8 Kampranis, S. C., Damianova, R., Atallah, M., Toby, G., Kondi, G., Tsichlis, P. N., and Makris, A. M. (2000) A novel plant glutathione *S*-transferase/peroxidase suppresses Bax lethality in yeast. J. Biol. Chem. **275**, 29207-29216

9 Barr, R. K. and Bogoyevitch, M. A. (2001) The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). IJBCB **33**, 1047-1063

10 Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes & Dev. **7**, 2135-2148

11 Gupta, S., Campbell, D., Dérijard, B., and Davis, R. J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science **267**, 389-393

12 Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) Integration of MAP kinase signal transduction pathways at the serum response element. Science **269**, 403-407

13 Xia, C. L., Cowell, I. G., Dixon, K. H., Pemble, S. E., Ketterer, B., and Taylor, J. B. (1991) Glutathione transferase π its minimal, promoter and downstream cis-acting element. Biochem. Biophys. Res. Comm. **176**, 233-240

14 Yokoyama, Y., Sagara, M., Sato, S., and Saito, Y. (1998) Value of glutathione S-transferase p and the oncogene products c-Jun, c-Fos, c-H-Ras, and c-Myc as a prognostic indicator in endometrial carcinomas. Gynecol. Oncol. **68**, 280-287

15 Adler, V., Yin, Z., Fuchs, S. Y., Benezra, M., Rosario, L., Tew, K. D., Pincus, M. R., Sardana, M., Henderson, C. J., Wolf, C. R., Davis, R. J., and Ronai, Z. (1999) Regulation of JNK signaling by GSTp. EMBO J. **18**, 1321-1334

16 Elsby, R., Kitteringham, N. R., Goldring, C. E., Lovatt, C. A., Chamberlain, M., Henderson, C. J., Wolf, C. R., and Park, B. K. (2003) Increased constitute c-Jun N-terminal kinase signaling in mice lacking glutathione *S*-transferase Pi. J. Biol. Chem.

17 Yin, Z., Ivanov, V. N., Habelhah, H., Tew, K., and Ronai, Z. (2000) Glutathione *S*-transferase p elicits protection against H_2O_2 -induced cell death via coordinated regulation of stress kinases. Cancer Res. **60**, 4053-4057

18 Cho, S.-G., Lee, Y. H., Park, H.-S., Ryoo, K., Kang, K. W., Park, J., Eom, S.-J., Kim, M. J., Chang, T.-S., Choi, S.-Y., Shim, J., Kim, Y., Dong, M.-S., Lee, M.-J., Kim, S. G., Ichijo, H., and Choi, E.-J. (2001) Glutathione *S*-transferase Mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. J. Biol. Chem. **276**, 12749-12755

19 Stronach, B. E. and Perrimon, N. (1999) Stress signaling in *Drosophila*. Oncogene **18**, 6172-6182

20 Holland, P. M., Suzanne, M., Campbell, J. S., Noselli, S., and Cooper, J. A. (1997) MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to *hemipterous*. J. Biol. Chem. **272**, 24994-24998

21 Sluss, H. K., Han, Z., Barrett, T., Davis, R. J., and Ip, Y. T. (1996) A JNK signal transduction pathway that mediates morphogenesis and immune response in *Drosophila*. Genes Develop. **10**, 2745-2758

22 Zhang, K., Chaillet, J. R., Perkins, L. A., and Halazonetis, T. D. (1990) *Drosophila* homolog of the mammalian *jun* oncogene is expressed during embryonic development and activates transcription in mammalian cells. Proc. Natl. Acad. Sci. USA **87**, 6281-6285

23 Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L., and Ketterman, A. J. (2001) Genomic organization and putative promotors of highly conserved glutathione S-transferases originating by alternative splicing in *Anopheles dirus*. Insect Biochem. Molec. Biol. **31**, 75-85

24 Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L., and Ketterman, A. J. (2001) Heterologous expression and characterization of alternatively spliced glutathione *S*-transferases from a single *Anopheles* gene. Insect Biochem. Molec. Biol. **31**, 867-875

25 Wolter, S., Mushinski, J. F., Saboori, A. M., Resch, K., and Kracht, M. (2001) Inducible expression of a constitutively active mutant of MAP kinase kinase (MKK) 7 specifically activates JUN N-terminal protein kinase (JNK), alters expression of at least nine genes, and inhibits cell proliferation. J. Biol. Chem.

26 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254

27 Rowe, J. D., Nieves, E., and Listowsky, I. (1997) Subunit diversity and tissue distribution of human glutathione S-transferase: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. Biochem. J. **325**, 481-486

28 Ferrell, J. E. Jr. (1996) Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. TIBS **21**, 460-466

29 Ding, Y., Ortelli, F., Rossiter, L. C., Hemingway, J., and Ranson, H. (2003) The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. BMC Genomics **4**, 35-50

30 Nurtdinov, R. N., Artamonova, I. I., Mironov, A. A., and Gelfand, M. S. (2003) Low conservation of alternative splicing patterns in the human and mouse genomes. Human Molecular Genetics **12**, 1313-1320

31 Pearson, G., Robinson, F., Gibson, T. B., Xu, B., Karandikar, M., Berman, K., and Cobb, M. H. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Reviews **22**, 153-183

32 Allardyce, C. S., McDonagh, P. D., Lian, L.-Y., Wolf, C. R., and Roberts, G. C. K. (1999) The role of tyrosine-9 and the C-terminal helix in the catalytic mechanism of Alpha-class glutathione S-transferases. Biochem. J. **343**, 525-531

33 Nilsson, L. O., Gustafsson, A., and Mannervik, B. (2000) Redesign of substrate-selectivity determining modules of glutathione transferase A1–1 installs high catalytic efficiency with toxic alkenal products of lipid peroxidation. Proc. Natl. Acad. Sci. USA **97**, 9408-9412

34 Pal, A., Gu, Y., Pan, S.-S., Ji, X., and Singh, S. V. (2001) C-terminal region amino acid substitutions contribute to catalytic differences between murine class alpha glutathione transferases mGSTA1-1 and mGSTA2-2 toward anti-diol epoxide isomers of benzo[c]phenanthrene. Biochemistry **40**, 7047-7053

35 Nuccetelli, M., Mazzetti, A. P., Rossjohn, J., Parker, M. W., Board, P., Caccuri, A. M., Federici, G., Ricci, G., and Lo Bello, M. (1998) Shifting substrate specificity of human glutathione transferase (from class pi to class alpha) by a single point mutation. Biochem. Biophys. Res. Comm. **252**, 184-189

36 Oakley, A. J., Rossjohn, J., Lo Bello, M., Caccuri, A. M., Federici, G., and Parker, M. W. (1997) The three-dimensional structure of the human pi class glutathione S-transferase P1-1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. Biochemistry **36**, 576-585

37 Oakley, A. J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A. J., and Wilce, M. C. J. (2001) The crystal structures of glutathione S-transferases isozymes 1–3 and 1–4 from *Anopheles dirus* species B. Protein Science **10**, 2176-2185

38 Ward, N. E., Pierce, D. S., Chung, S. E., Gravitt, K. R., and O'Brian, C. A. (1998) Irreversible inactivation of protein kinase C by glutathione. J. Biol. Chem. **273**, 12558-12566

39 Liu, B. and Hannun, Y. A. (1997) Inhibition of the Neutral Magnesium-dependent Sphingomyelinase by Glutathione. J. Biol. Chem. **272**, 16281-16287

40 Fournier, D., Bride, J.-M., Poirié, M., Bergé, J.-B., and Plapp, F. W. (1992) Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. J. Biol. Chem. **267**, 1840-1845

41 Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M. V., Unger, M., Collins, F. H., and Feyereisen, R. (2002) Evolution of supergene families associated with insecticide resistance. Science **298**, 179-181

42 Sharrocks, A. D., Yang, S.-H., and Galanis, A. (2000) Docking domains and substrate specificity determination for MAP kinases. TIBS **25**, 448-453

43 Tanoue, T., Maeda, R., Adachi, M., and Nishida, E. (2001) Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. EMBO J. **20**, 466-479

44 Adler, V., Pincus, M. R., Minamoto, T., Fuchs, S. Y., Bluth, M. J., Brandt-Rauf, P. W., Friedman, F. K., Robinson, R. C., Chen, J. M., Wang, X. W., Harris, C. C., and Ronai, Z. (1997) Conformation-dependent phosphorylation of p53. Proc. Natl. Acad. Sci. USA **94**, 1686-1691

45 Wang, T., Arifoglu, P., Ronai, Z., and Tew, K. D. (2001) Glutathione *S*-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. J. Biol. Chem. **276**, 20999-21003

46 Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nature Cell Biology **2**, 110-116

47 Ma, B., Elkayam, T., Wolfson, H., and Nussinov, R. (2003) Protein–protein interactions: Structurally conserved residues distinguish between binding sites and exposed protein surfaces. Proc. Natl. Acad. Sci. USA **100**, 5772-5777

48 Hu, Z., Ma, B., Wolfson, H., and Nussinov, R. (2000) Conservation of polar residues as hot spots at protein interfaces. Proteins **39**, 331-342

49 Mever, M., Wilson, P., and Schomburg, D. (1996) Hydrogen bonding and molecular surface shape complementarily as a basis for protein docking. J. Mol. Biol. **264**, 199-210

50 Hansson, L. O., Bolton-Grob, R., Massoud, T., and Mannervik, B. (1999) Evolution of differential substrate specificities in mu class glutathione transferases probed by DNA shuffling. J. Mol. Biol. **287**, 265-276

TABLE 1

The HEP and JNK proteins affect on the GST activity

The recombinant proteins of HEP and JNK were incubated with the different GST splice forms in 1:1 molar ratio for 5 min at room temperature. The % inhibition of GST activity was calculated using reaction that contained no kinase proteins as the control. No inhibition of GST activity of GSTD1-1 by JNK protein was detected.

GSTs	Inhibition of GSTs by HEP (%)	Inhibition of GSTs by JNK (%)
D1-1	54.64 ± 3.83	No inhibition detected
D2-2	53.29 ± 7.71	85.35 ± 6.72
D3-3	23.71 ± 1.88	44.64 ± 6.92
D4-4	29.27 ± 2.99	68.45 ± 4.00

The data are mean ± standard deviation from at least four independent experiments

TABLE 2

The mechanism of GST and kinase protein interaction

The type of inhibition and affinity binding (K_i) of GST and kinase protein interaction were studied by varying concentration of CDNB from 0.05 to 3.0 and measuring kinetic parameters under the standard conditions for each GST enzyme.

GST	Type of Inhibition	K_i (nM) HEP-GST	K_i (nM) JNK-GST
D1-1	Non-competitive	50.0 ± 2.84	Not determined
D2-2	Non-competitive	21.3 ± 5.18	73.9 ± 20.1
D3-3	Non-competitive	43.6 ± 7.19	76.8 ± 15.4
D4-4	Non-competitive	125.0 ± 45.2	218 ± 86.3

The data are means ± standard error for at least four independent experiments.

TABLE 3

Specific activity of GST splice forms using various hydrophobic substrates

GSTs	Specific activity (μmol/min/mg of protein)			
	CDNB	DCNB	PNBC	PNPB
D1-1	6.54 ± 0.53	0.070 ± 0.002	1.05 ± 0.191	0.002 ± 0.002
D2-2	45.1 ± 3.41	0.177 ± 0.006	17.1 ± 1.09	0.047 ± 0.010
D3-3	67.5±1.97	0.312 ± 0.023	2.96 ± 0.292	0.002 ± 0.007
D4-4	41.8 ± 1.40	0.042 ± 0.011	2.73 ± 0.105	0.023 ± 0.002

The data are means ± standard error for at least five separate assays. The substrate concentrations used were: CDNB 1 mM; DCNB 1 mM; *p*-nitrobenzyl chloride (PNBC) 1.2 mM and *p*-nitrophenyl bromide (PNPB) 0.1 mM.

FIGURE LEGENDS

Figure 1. GST spliceforms are both positive and negative regulators of JNK activity

A) The experiment was performed using GST: HEP_{3E}: JNK: Jun 1-104 in 10:1:2:10 molar ratio. GST was incubated with constitutively active HEP_{3E} for 10 min at room temperature before adding JNK and Jun with 3 μCi of [γ-^{32}P] ATP for 25 min of phosphorylation time at room temperature. Proteins were then separated by SDS-PAGE. and JNK activity was visualized by autoradiography of the ^{32}P-labeled Jun. The top panel is an autoradiograph showing Jun phosphorylation in the presence and absence of different GST isoforms. The bottom panel is an SDS-PAGE showing the sample loading for Jun. The histogram quantifies the effects of GSTs on Jun phosphorylation when compared to the reaction in the absence of these GSTs. The figures shown are representative of at least three independent experiments.

B) The experiment was performed using GST: HEP_{3E}: JNK in 10:1:2 molar ratio. GST was incubated with constitutively active HEP_{3E} for 10 min at room temperature before adding JNK with 3 μCi of [γ-^{32}P] ATP for 25 min of phosphorylation time at room temperature. Proteins were then separated by SDS-PAGE, and kinase activity was visualized by autoradiography of the ^{32}P-labeled substrates. The JNK protein was a substrate for HEP as well as HEP was a substrate for JNK (21). The autoradiograph in the top panel shows HEP and JNK phosphorylation in the presence and absence of different GST isoforms. The bottom panel is an SDS-PAGE showing the sample loading for HEP and JNK. The histogram quantifies the effects of the GSTs on HEP and JNK phosphorylation. The figures shown are representative of at least three independent experiments.

Figure 2. JNK changes the substrate specificity of GST spliceforms

GST and JNK were incubated in 1:1 molar ratio for 5 min at room temperature. The GST activity was measured using various hydrophobic co-substrates. The % activity change of GST activity was determined by comparing the reactions in the presence and absence of JNK. The specific activity of each GST for the substrates in the absence of JNK is shown in Table 3. There was no activity change in GSTD1-1 activity using CDNB as the co-

substrate due to the lack of a JNK effect (Table 1). The data shown are representative of at least four independent experiments with similar results.

Figure 3. The presence of glutathione changed the JNK effects on GST activity

The effects of JNK on GST were determined in the absence and presence of GSH. GST and JNK were incubated in 1:1 molar ratio in the presence and absence of 2 mM GSH for 5 min at room temperature. The GST activity was measured using various hydrophobic co-substrates. The % activity change of GST activity was then calculated, Panel A represents the specific activity of GSTD1-1 and D2-2 affected by JNK and was employed for comparison with the reaction containing GSH, shown in Panel B.

Figure 4. The presence of glutathione changed the GST affects on JNK activity

GST: HEP: JNK: Jun 1-104 in 10:1:2:10 molar ratio were incubated in the presence and absence of 10 mM GSH with 3 μCi of [γ-^{32}P] ATP for 25 min of phosphorylation time at room temperature. *A)* The autoradiograph in the top panel shows JNK phosphorylation in the presence and absence of GSH. The bottom panel is an SDS-PAGE showing the sample loading for JNK. The histogram quantifies in fold activity the GSTs effect on JNK phosphorylation. *B)* The autoradiograph in the top panel shows Jun phosphorylation in the presence and absence of GSH. The bottom panel is an SDS-PAGE showing the sample loading for Jun. The histogram quantifies the GSTs' effects on Jun phosphorylation. The figures shown are representative of at least three independent experiments.

Figure 5. A proposed mechanism of GST regulation of stress kinase proteins through a dissociation/association process

The different isoforms of GSTs possess different JNK effector properties. GST_I refers to JNK inhibitor and GST_A represents a JNK activator. The JNK regulation occurs upon stress. *A)* In normal conditions, GST_I inhibits JNK and maintains JNK at basal level activity (10). *B)* Once stress occurs, a mechanism to dissociate GST_I from JNK comes into effect. GST_A may now associate with JNK or an upstream activating kinase and increase or modulate the kinase cascade response.

Figure 1.

A.

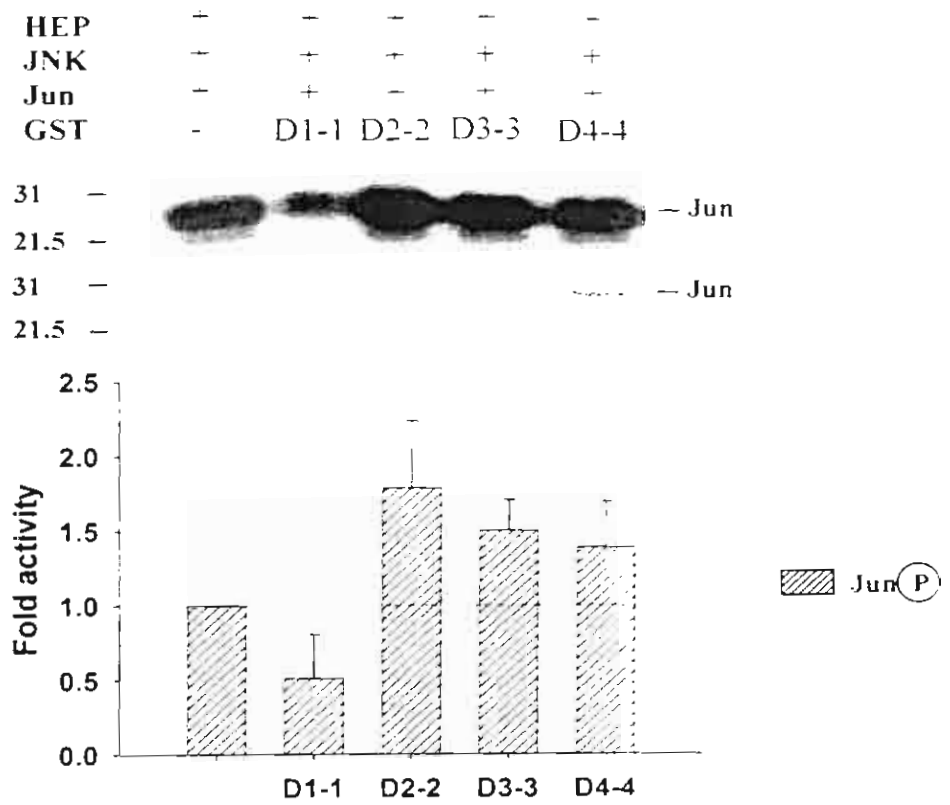

HEP
JNK
Jun
GST
-
D1-1
D2-2
D3-3
D4-4
31
21.5
31
21.5
Jun
Jun
Fold activity
2.5
2.0
1.5
1.0
0.5
0.0
D1-1
D2-2
D3-3
D4-4
Jun(P)

Figure 1.

B.

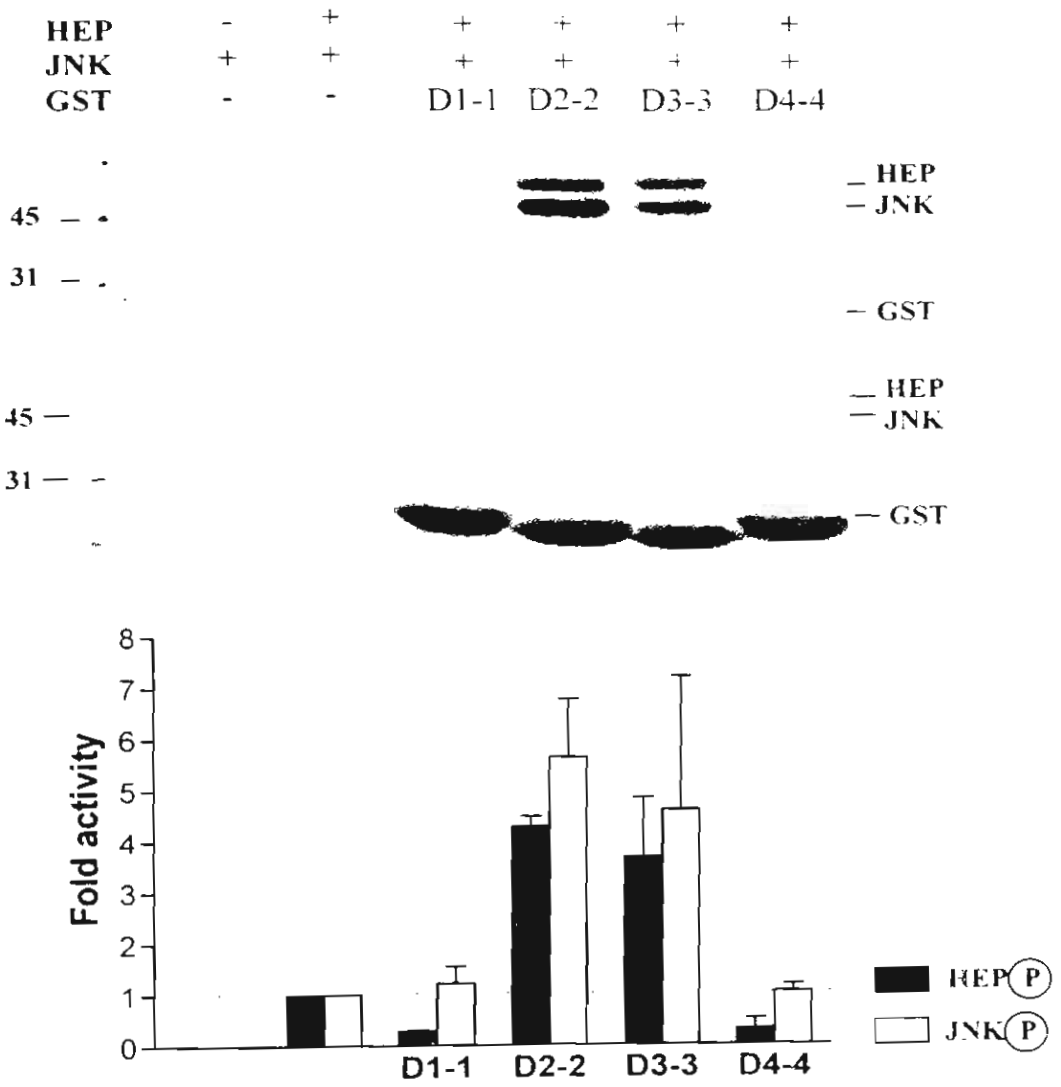

HEP - + + + + +
JNK + + + + + +
GST - - D1-1 D2-2 D3-3 D4-4
45
31
HEP
JNK
GST
HEP
JNK
GST
45
31
Fold activity
0
1
2
3
4
5
6
7
8
D1-1
D2-2
D3-3
D4-4
HEP(P)
JNK(P)

Figure 2.

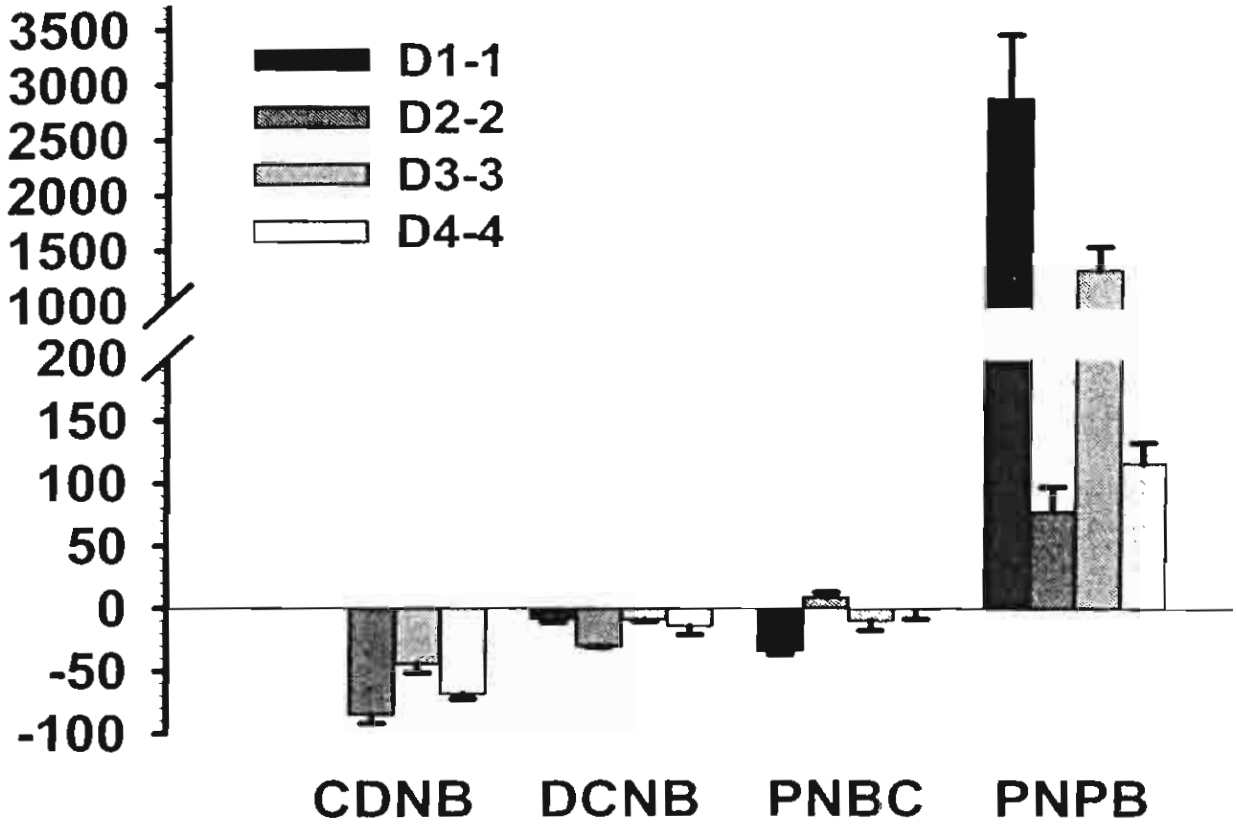
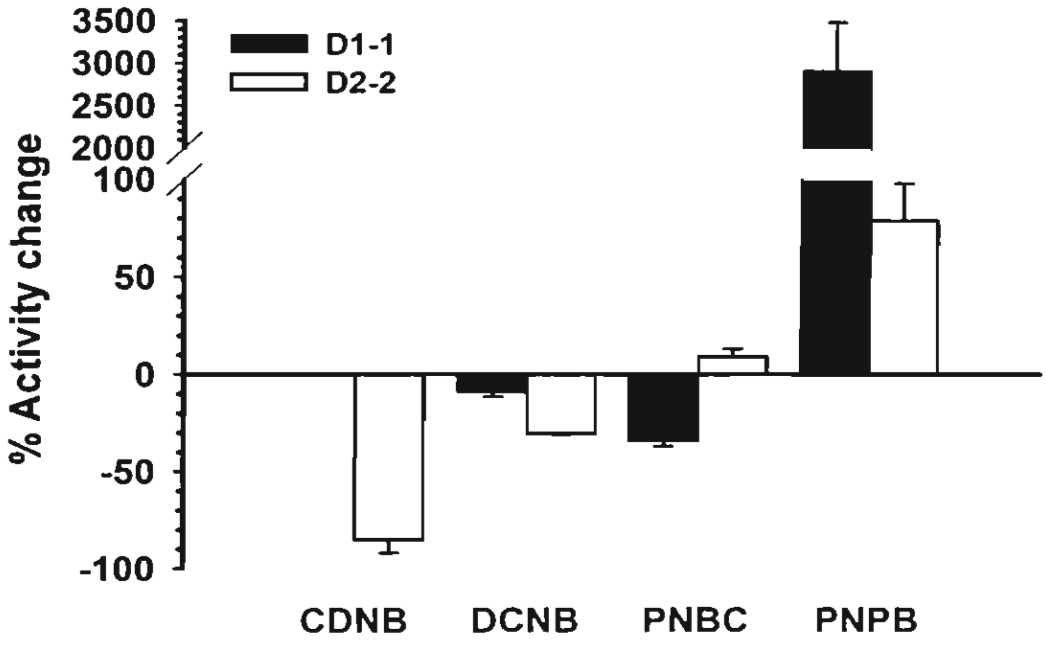

3500
3000
2500
2000
1500
1000
200
150
100
50
0
-50
-100
D1-1
D2-2
D3-3
D4-4
CDNB
DCNB
PNBC
PNPB

Figure 3.

A.


3500
3000
2500
2000
100
50
0
-50
-100
% Activity change
D1-1
D2-2
CDNB
DCNB
PNBC
PNPB

B.

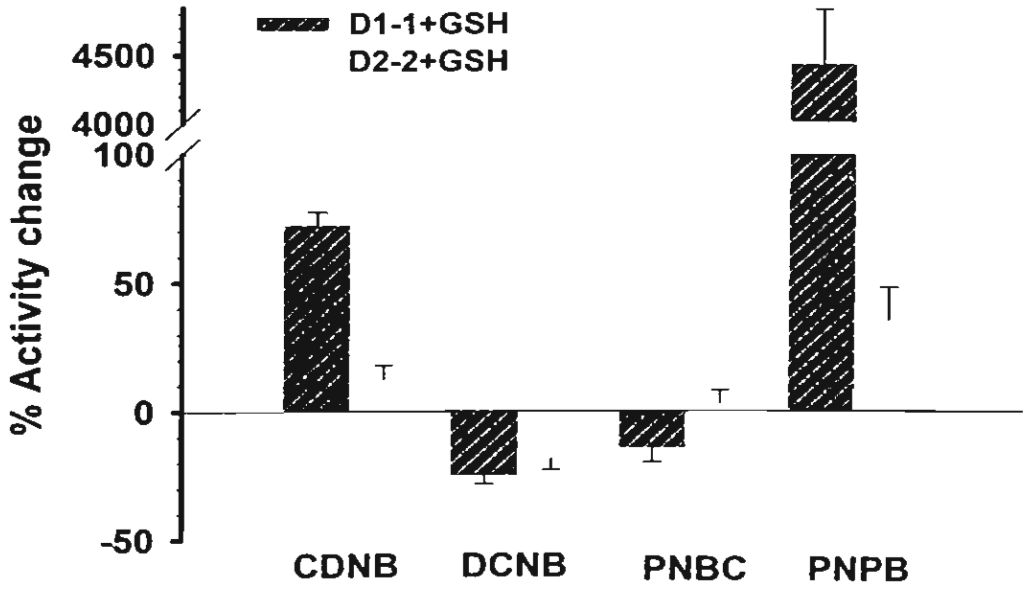
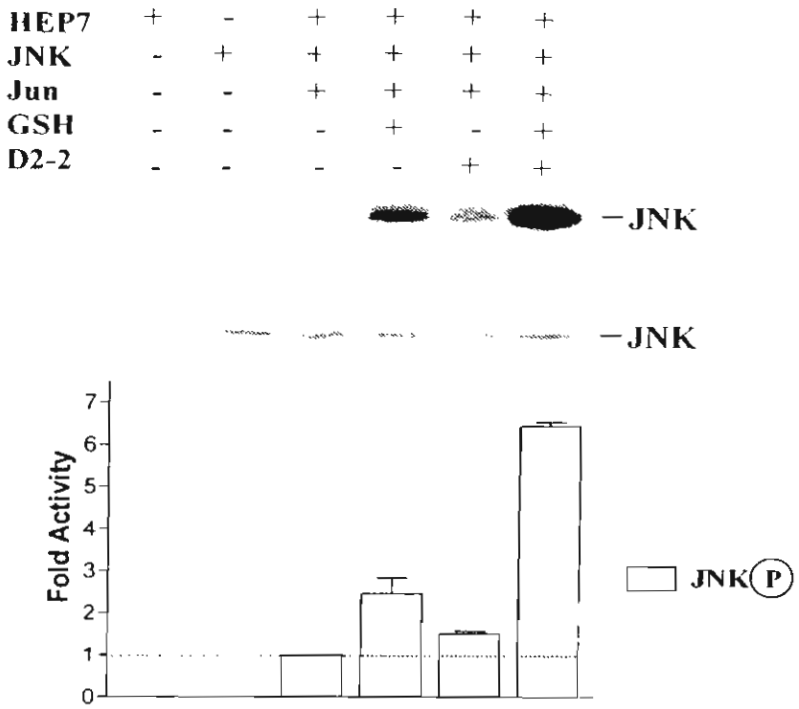

4500
4000
100
50
0
-50
% Activity change
D1-1+GSH
D2-2+GSH
CDNB
DCNB
PNBC
PNPB

Figure 4.

A.


HEP7 + - + + + +
JNK - + + + + +
Jun - - + + + +
GSH - - - + - +
D2-2 - - - - + +
—JNK
—JNK
Fold Activity
0
1
2
3
4
5
6
7
JNK(P)

B.

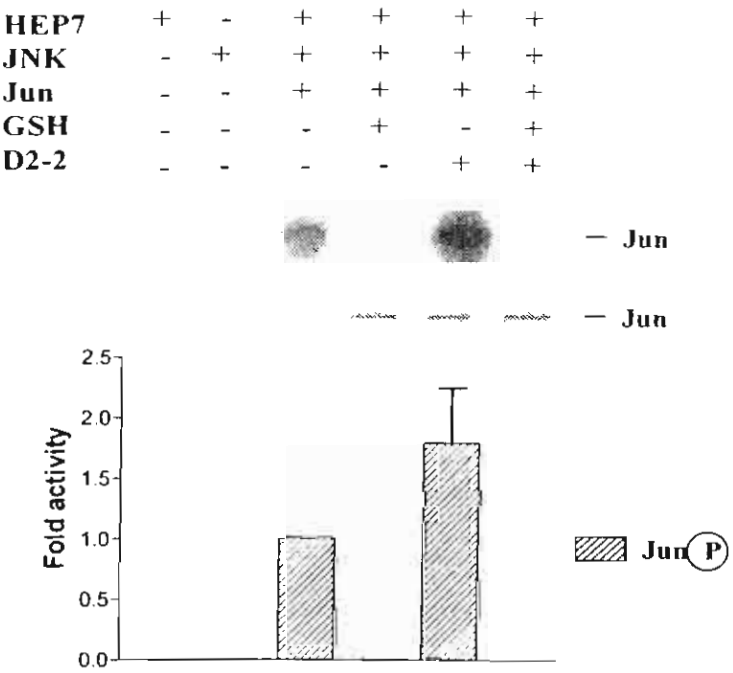
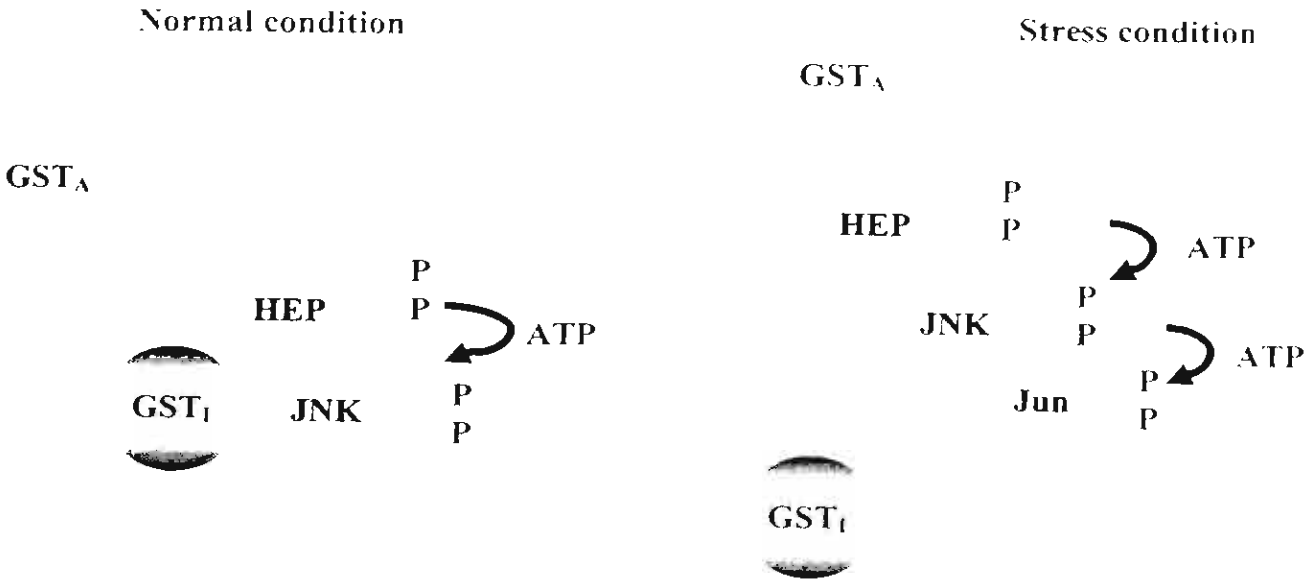

HEP7 + - + + + +
JNK - + + + + +
Jun - - + + + +
GSH - - - + - +
D2-2 - - - - + +
— Jun
— Jun
Fold activity
0.0
0.5
1.0
1.5
2.0
2.5
Jun(P)

Figure 5.


Normal condition
Stress condition
GST$_A$
GST$_A$
HEP
P
P
ATP
JNK
P
P
GST$_I$
JNK
P
P
HEP
P
P
ATP
P
P
JNK
ATP
Jun
P
P
GST$_I$

Catalytic and structural contributions for glutathione-binding residues in a Delta class glutathione S-transferase

Pakorn WINAYANUWATTIKUN and Albert J. KETTERMAN[1]

Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand

Glutathione S-transferases (GSTs) are dimeric proteins that play a major role in cellular detoxification. The GSTs in mosquito *Anopheles dirus* species B, an important malaria vector in South East Asia, are of interest because they can play an important role in insecticide resistance. In the present study, we characterized the *Anopheles dirus* (Ad)GST D3-3 which is an alternatively spliced product of the *adgst1* AS1 gene. The data from the crystal structure of GST D3-3 shows that Ile-52, Glu-64, Ser-65, Arg-66 and Met-101 interact directly with glutathione. To study the active-site function of these residues, alanine substitution site-directed mutagenesis was performed resulting in five mutants: I52A (Ile-52 → Ala), E64A, S65A, R66A and M101A. Interestingly, the E64A mutant was expressed in *Escherichia coli* in inclusion bodies, suggesting that this residue is involved with the tertiary structure or folding property of this enzyme. However, the I52A, S65A, R66A and M101A mutants were purified by glutathione affinity chromatography and the enzyme activity characterized. On the basis of steady-state kinetics, difference spectroscopy, unfolding and refolding studies, it was concluded that these residues: (1) contribute to the affinity of the GSH-binding site ('G-site') for GSH, (2) influence GSH thiol ionization, (3) participate in k_{cat} regulation by affecting the rate-limiting step of the reaction, and in the case of Ile-52 and Arg-66, influenced structural integrity and/or folding of the enzyme. The structural perturbations from these mutants are probably transmitted to the hydrophobic-substrate-binding site ('H-site') through changes in active site topology or through effects on GSH orientation. Therefore these active site residues appear to contribute to various steps in the catalytic mechanism, as well as having an influence on the packing of the protein.

Key words: active site, *Anopheles dirus*, glutathione S-transferase, GSH-binding site, structure effect.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a widely distributed family of detoxifying dimeric enzymes found in most forms of life (e.g. vertebrates, plants, insects, yeasts and aerobic bacteria) [1,2]. GSTs catalyse the conjugation of hydrophobic substrates, such as drugs, herbicides and insecticides, with electrophilic centres to GSH [3,4]. The conjugation of GSH to such molecules increases their solubility and facilitates further metabolic processing [1,5–7]. In addition, these enzymes also carry out a range of other functions. They have peroxidase [5,8] and isomerase activity [9], they can inhibit the Jun N-terminal kinase (thus protecting cells against H_2O_2-induced cell death) [10], and they are able to non-catalytically bind a wide range of endogenous and exogenous ligands [11–13].

All cytosolic GSTs have the same basic protein folding, which comprises two domains. The N-terminal domain (domain I) adopts a α/β topology and provides the GSH-binding site (G-site) [3,14]. It is currently believed that the residues which contribute to binding glutathione involve a network of specific polar interactions between GSH and G-site residues that are either conserved or conservatively replaced between classes. The C-terminal domain (domain II) is an all-helical structure and provides the structural element for recognition of the broad range of hydrophobic co-substrate [H-site (hydrophobic-substrate-binding site)], which lies adjacent to the G-site [3,14]. It shows the greatest variability across the GST classes [15–20] and helps to define the substrate selectivity of the enzyme [3,14,21].

The enzyme catalysis of nucleophilic aromatic substitution reactions can be divided into several steps, involving substrate binding, activation of GSH by promoting and stabilizing the anionic thiol group, nucleophilic attack by the anionic glutathione to the hydrophobic substrate possessing an electrophilic centre, product formation and finally product release [22–25]. The function of several active site residues have been elucidated: Ser-9 is involved in the production and stabilization of the ionized GSH; Tyr-113 is involved in the formation and release of the GSH conjugate of *Lucilia cuprina* GST [24]; Cys-47 acts as a molecular switch for different conformations of human (h)GST P1-1 [26]; His-40, Lys-41 and Gln-53 play roles in GSH binding and the structural integrity of *Zea mays* GSTI [27]. However, there is still a need to determine the contribution of other important active site amino acids.

The *Anopheles dirus adgst1AS1* gene is spliced to yield 4 different isoforms; D1, D2, D3 and D4 [28,29]. The present study focuses on AdGST D3, because it has high expression levels in *Escherichia coli*, displays high enzymic activity and the crystal structure is available [28,30]. The aim of this work is to characterize the function of the glutathione-binding residues in adGST D3-3. Five residues in the G-site which are shown in the crystal structure to directly interact with the glutathione were investigated. The residues Ile-52, Glu-64 and Ser-65 are conserved among the GST classes, whereas Arg-66 and Met-101 are variable across the GST classes (Figure 1). These residue positions were substituted with alanine and characterized for both structural and catalytic roles. The results showed that these active site residues

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; FDNB, 1-fluoro-2,4-dinitrobenzene; G-site, GSH-binding site; GST, glutathione S-transferase; AdGST, *Anopheles dirus* GST; hGST, human GST; rGST, rat GST; H-site, hydrophobic-substrate-binding site; I52A, Ile-52 → Ala substitution etc.

[1] To whom correspondence should be addressed (email frakt@mahidol.ac.th).

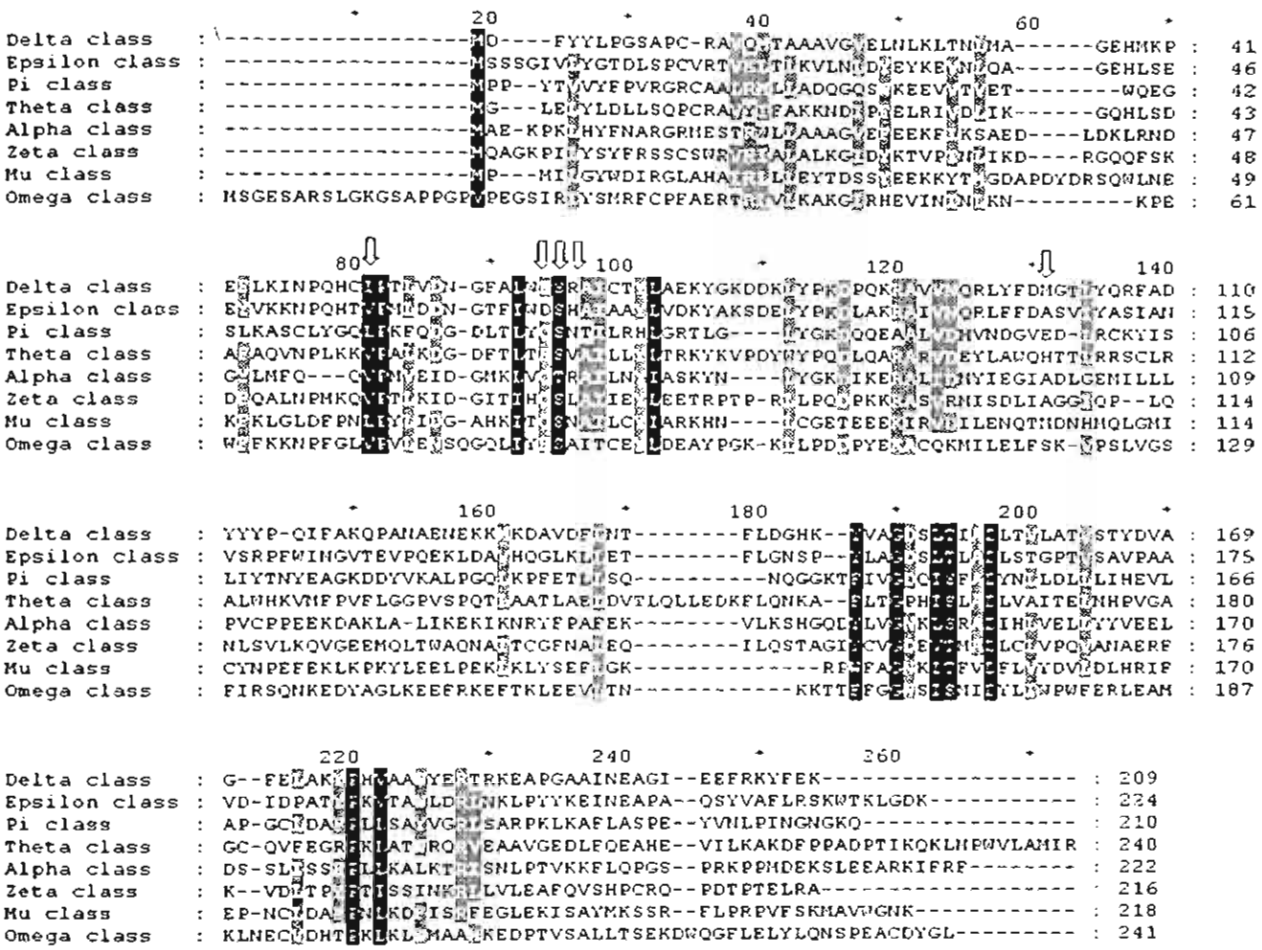


Figure 1 Amino acid alignments of several classes of GSTs

Gaps introduced to maximize sequence similarity are shown by a horizontal dash. Black shadings represent 100 % sequence similarity and gray shadings represent 80 % sequence similarity. Vertical arrows indicate equivalent position of Ile-52, Glu-64, Ser-65, Arg-66 and Met-101 in Delta class. (GenBank® accession numbers are AF273039, NP_611323.1, A37378, NP_000844.1, NP_665683.1, O43708, NP_000552.1 and AH004172.1 respectively)

make contributions not only to enzyme activity, but also to physical properties.

EXPERIMENTAL

Expression plasmid and site-directed mutagenesis

The plasmid pET3a-adgstD3, as previously described [28], was used to generate the active site residue mutants via PCR-based site-directed mutagenesis. The selected residues Ile-52, Glu-64, Ser-65, Arg-66 and Met-101, which interact directly with GSH, were substituted with alanine by using the mutagenic primers that have been designed according to the 5′ and 3′ sequence of the AdGST D3 wild-type gene (GenBank® accession number AF273039). Each mutant was randomly screened by restriction digestion analysis. Mutant plasmids could be distinguished from the template by digestion with the restriction enzyme corresponding to the restriction recognition site introduced by the mutagenic primers. The full-length GST coding sequence of the plasmids carrying the I52A, E64A, S65A, R66A and M101A mutations were verified by the dideoxy-chain-termination method.

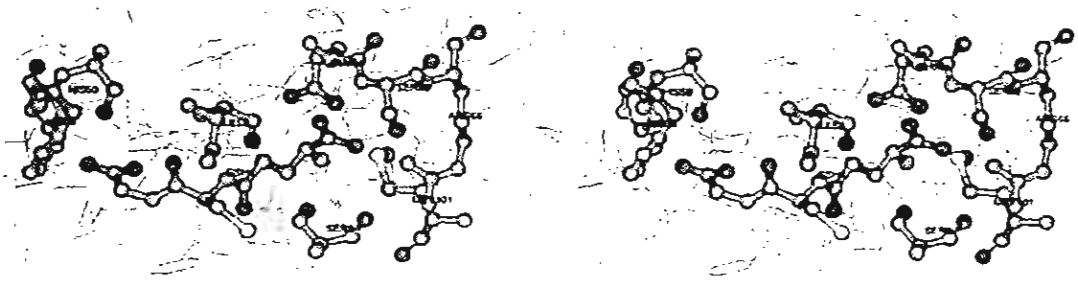
Protein expression and purification

E. coli cells, containing wild-type and mutant plasmid pET3a-adgstD3, were grown in Luria–Bertani medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The expression of GST was induced by the addition of 0.1 mM isopropyl 1-thio-β-galactopyranoside when D_{600} was 0.5. Following induction for 3 h, cells were harvested by centrifugation at 5000 *g* at 4 °C for 10 min. The soluble recombinant GST proteins were purified by GSTrap affinity chromatography (Amersham Pharmacia) or S-hexylglutathione agarose (Sigma) affinity chromatography in the case of low affinity towards the glutathione ligand [31]. Glycerol was then added to a final concentration of 50 % (v/v) and the purified concentrated GSTs stored at −20 °C. After purification, the enzymes were homogeneous, as determined by SDS/PAGE. The protein concentration was determined by the Bradford method using BSA as a standard [32].

Kinetic studies

Steady-state kinetics were studied for wild-type and mutant enzymes at various concentrations of CDNB (1-chloro-2,4-dinitrobenzene) and GSH in 0.1 M phosphate buffer, pH 6.5. The reaction was monitored at 340 nm, $\varepsilon = 9600\ M^{-1} \cdot cm^{-1}$. Apparent kinetic parameters, k_{cat}, K_m and k_{cat}/K_m were determined by fitting the collected data to a Michaelis–Menten equation by non-linear regression analysis using GraphPad Prism (GraphPad software, San Diego, CA, U.S.A.).

The pH dependence of k_{cat}/K_m^{CDNB} was obtained by using 0.1 M sodium acetate buffers (from pH 5.0 to pH 5.5) and 0.1 M potassium phosphate buffer (from pH 6.0 to pH 8.5).



gure 2 Stereo view of the studied residues around the G-site of adGST D3-3

-52, Glu-64, Ser-65, Arg-66 and Met-101 interact directly with GSH.

able 1 Steady-state kinetic parameters and pK_a values for the thiol group of GSH of wild-type and mutants of adGSTD3-3 for the CDNB conjugation reaction t pH 6.5 and 25 °C

he enzyme activities were measured at varying concentrations of CDNB and GSH in 0.1 M phosphate buffer, pH 6.5. The pK_a was obtained by using 0.1 M sodium acetate buffers (from pH 5.0 to H 5.5) and 0.1 M potassium phosphate buffer (from pH 6.0 to pH 8.5). The reaction was monitored at 340 nm, $\varepsilon = 9600\ M^{-1}\ cm^{-1}$

Enzyme	k_{cat} (s^{-1})	K_m^{GSH} (mM)	K_m^{CDNB} (mM)	k_{cat}/K_m^{GSH} (s^{-1}/mM)	k_{cat}/K_m^{CDNB} (s^{-1}/mM)	pK_a
Wild-type	35.4	0.27 ± 0.05	0.14 ± 0.01	130.69	245.95	6.36 ± 0.11
I52A	16.9	6.50 ± 0.61	0.39 ± 0.03	2.60	43.72	6.78 ± 0.14
S65A	29.8	1.22 ± 0.12	0.34 ± 0.06	24.50	87.18	6.89 ± 0.23
R66A	3.3	5.10 ± 0.40	0.22 ± 0.04	0.64	14.59	7.23 ± 0.18
M101A	63.0	1.08 ± 0.09	0.19 ± 0.01	58.48	337.12	6.10 ± 0.07

The second order kinetic constants at pH 6.5 for the spontaneous reaction of GSH with CDNB and FDNB (1-fluoro-2,4-dinitrobenzene), and the catalytic-centre activities ('turnover numbers') at pH 6.5 for adGST D3-3 with CDNB and FDNB as co-substrates, were obtained as described previously [33].

The effect of viscosity on kinetic parameters was assayed by using 0.1 M potassium phosphate buffer, pH 6.5, with various glycerol concentrations. Viscosity values (η) at 25 °C were calculated as described previously [34].

The specific activities of the enzymes were determined using a spectrophotometer with five different substrates: CDNB, 1,2-dichloro-4-nitrobenzene, ethacrynic acid, *p*-nitrophenethyl bromide and *p*-nitrobenzyl chloride as described previously [35,36].

Structural studies

One of the structural studies performed was to determine a half-life stability for the GST protein at 45 °C. The wild-type and mutant enzymes were incubated at 45 °C at a protein concentration of 1 mg/ml. The inactivation time courses were determined by taking suitable aliquots at the different time points for assay of remaining activity to calculate half-life of the enzyme [36].

Spectroscopic properties of the wild-type and mutant proteins were also studied. Intrinsic fluorescence emission spectra were measured at the excitation wavelength 295 nm, and the λ_{max} and the fluorescence intensity of emission spectra were analysed at a protein concentration of 0.2 mg/ml [37].

A refolding experiment was also performed with the enzymes first being denatured in 4 M guanidinium chloride in renaturation buffer (0.2 M phosphate, 1 mM EDTA and 5 mM DTT, pH 7.0) at 25–27 °C for 30 min and then rapidly diluted (defining time 0) 1:40 into renaturation buffer. Therefore the final guanidinium chloride concentration was 0.1 M during refolding. Recovered activity was monitored as a function of time by taking appropriate aliquots of the renaturation mixture and immediately assaying for activity. Refolding rate constants were determined by non-linear regression analysis using a single exponential equation [37].

RESULTS AND DISCUSSION

Protein expression and purification

Five residues, shown by crystal structure to directly interact with glutathione, were studied to characterize their roles in catalysis and structure (Figure 2). These residues, Ile-52, Glu-64, Ser-65, Arg-66 and Met-101 in adGSTD3-3, were individually replaced with alanine by oligonucleotide-directed mutagenesis. The wild-type and mutant enzymes were expressed as soluble forms, except for I52A and E64A, which were insoluble at 37 °C. Decreasing the expression temperature to 25 °C allowed the I52A mutant protein to be expressed in a soluble form. However, the GST with the E64A substitution was still expressed as an insoluble form, even at 18 °C. Attempts at refolding the E64A protein were unsuccessful. This suggests that Glu-64 is a critical residue involved in the initial packing or folding of the protein to yield the final tertiary structure. The soluble GSTs were purified and gave a single band on SDS/PAGE. In each case, the unbound activity from the GSH affinity chromatography of the recombinant GSTs varied from 2–25% of the total activity, displaying a reduction in binding to the chromatography media.

Steady-state kinetics

Steady-state kinetics were performed with various concentrations of glutathione and CDNB as substrates. The reactions followed Michaelis–Menten kinetics, and the kinetic parameters k_{cat} and K_m were determined by non-linear regression analysis (Table 1). All of the mutations increased K_m values for glutathione, especially for I52A and R66A, which had values 24- and 19-fold greater than wild-type, demonstrating a decreased affinity toward glutathione. However, the mutations yielded only slight differences in the affinity of binding towards the substrate CDNB when compared with the wild-type enzyme.

The differences in k_{cat} value in the nucleophilic aromatic substitution reaction with CDNB observed for I52A and R66A decreased approx. 2- and 10-fold respectively, whereas the

Table 2 Effect of fluoride/chloride leaving group substitution on the rate of catalysis

The catalytic-centre activities of the conjugation reaction catalyzing by adGST D3-3 enzymes of GSH with CDNB and FDNB as co-substrates were calculated at pH 6.5

Enzyme	k_{cat}^{FDNB} (s^{-1})	k_{cat}^{CDNB} (s^{-1})
Wild-type	85.1	35.4
I52A	27.5	16.9
S65A	157.3	29.8
R66A	18.1	3.3
M101A	265.9	63.0

catalytic-centre activity for the M101A mutant is about 2 times greater than wild-type (Table 1). The question arose of whether the changes in k_{cat} value were due to changes in the activation of the GSH substrate bound in binary complex with the enzyme. The pH dependence of the kinetic parameters in the binary complex was determined to give the pK_a values in Table 1. The pK_a of the R66A mutant was about 1 pH unit higher than that found for the wild-type. This increased pK_a was also observed for a conserved hydroxy side-chain amino acid, Ser-9 in Delta class and tyrosine in Alpha, Mu and Pi classes, which play a important role in promoting and stabilizing anionic glutathione [3,23,24,38]. Several reports have shown that the glutamyl α-carboxylate of glutathione contributes to the ionization of the glutathione thiol group [39,40]. Arg-66 interacts directly with the glutamyl α-carboxylate of glutathione and appears to influence the ionization process of glutathione in Delta class GST. The involvement of positively charged residues in regulation of the electrostatic field also has been observed with other GSTs. For example, Arg-107 from hGST M2-2 [41] and Arg-15 hGST A1-1 [42] have been shown to contribute to active site ionization. For the other residues there are only slight differences in the pK_a of glutathione for the I52A, S65A and M101A GSTs, suggesting these residues may contribute to glutathione orientation to yield a suitable position for conjugation with the electrophilic substrate.

Determining the rate-limiting step in the adGST-D3-3-catalysed reaction

Normally, the reaction of nucleophilic aromatic substitution proceeds via a σ-complex intermediate. Substitution of the chlorine leaving group in the CDNB molecule by a more electronegative fluorine increases the second order rate constant of the spontaneous reaction with glutathione about 50-fold, indicating that the rate-limiting step is the σ-complex formation [24]. Therefore the effect on the catalytic-centre activity of the fluorine substitution for the chlorine in CDNB was examined. The results show that the k_{cat} does vary for the different GSTs (Table 2). This is most likely due to packing changes yielding a different architecture of the active site of the enzyme that responds differently to the smaller fluorine leaving group. However, this data indicates that the σ-complex formation is not the rate-limiting step. Therefore, the rate-limiting step in the enzymic reaction appears to be a physical step, rather than a chemical step.

Next we examined the effect of viscosity on the kinetic parameters to determine the rate-limiting step of the catalytic reaction. A decrease of the rate constant by increasing the medium viscosity would reflect the influence of diffusion on catalysis [43]. It would indicate that the rate-limiting step of the reaction is related to the product release or the diffusion-controlled structural transition of the protein. A plot of the reciprocal of the relative catalytic constant (k_{cat}^{o}/k_{cat}) against the relative viscosity (η/η^{o}) should

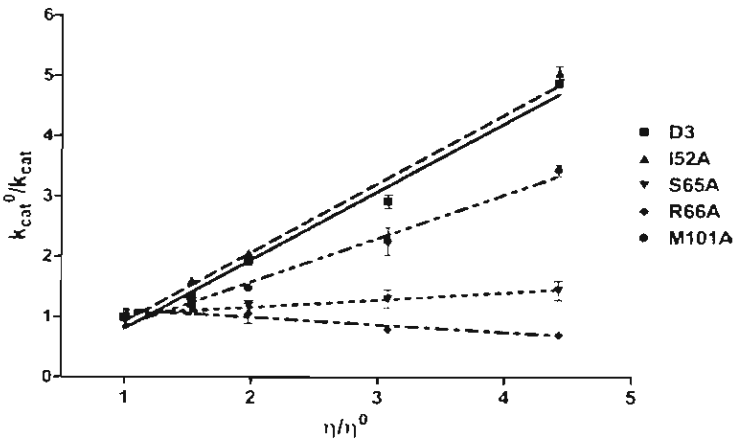


Figure 3 Viscosity effect on kinetic parameters of wild-type and mutant enzymes

The effect of viscosity on kinetic parameters was assayed by using 0.1 M potassium phosphate buffer, pH 6.5, with various glycerol concentrations. Dependence of the reciprocal of the relative catalytic-centre activity (k_{cat}^{o}/k_{cat}) on the relative viscosity (η/η^{o}) for CDNB as co-substrate D3, wild-type

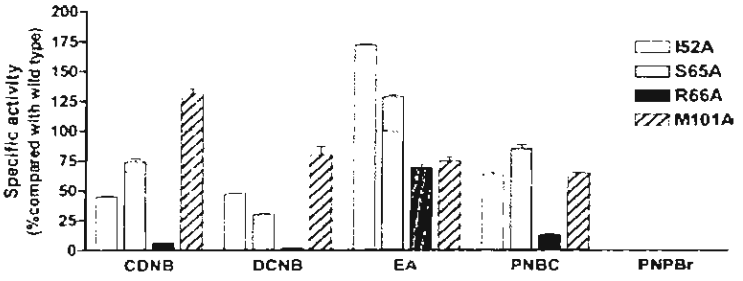


Figure 4 Substrate specific activity as a percentage change compared with the adGSTD3-3 (wild-type)

Five substrates CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), *p*-nitrophenethyl bromide (PNPBr) and *p*-nitrobenzyl chloride (PNBC) were used for enzyme activity assays

be linear. The slope should be equal to unity when the product release or structural transition is limited by a strictly diffusional barrier. If the slope approaches zero the chemistry or another non-diffusion barrier is rate-limiting. As shown in Figure 3 the inverse relative rate constant for the enzyme-catalysed reaction shows a linear dependence on the relative viscosity with a slope very close to unity for the GST D3-3 wild-type (1.14 ± 0.01), similar to the I52A mutant GST (1.16 ± 0.05). Diffusion-controlled motions of the protein have been reported to modulate the catalysis of other GST isoenzymes: a conformational change in the case of ternary complex formation of hGST P1-1 [33] and product release for rat (r)GST M1-1 [43], *L. cuprina* GST [24], rGST T2-2 [44] and Alpha-class GSTs [45]. In contrast the mutants S65A (0.12 ± 0.09), R66A (-0.12 ± 0.01) and M101A (0.73 ± 0.08) exhibited k_{cat} values with different degrees of viscosity dependence compared with the wild-type (Figure 3). In particular, the slopes of for the S65A and R66A mutants are very close to zero, indicating that the mutation changed the rate-limiting step of the enzyme from a physical to a non-physical step.

Substrate specificity

Substrate specificity determination revealed differences in the specificity or the interaction of the enzymes with several hydrophobic substrates (Figure 4). This data shows that mutations of the

Table 3 Thermal stability of wild-type and mutants of adGSTD3-3 at 45 °C

The wild-type and mutant enzymes were incubated at 45 °C at the protein concentration of 1 mg/ml. The inactivation time courses were determined by taking suitable aliquots at the different time points for assay of remaining activity to calculate half-life of the enzyme

Enzyme	Half-life at 45 °C (min^{-1})
Wild-type	2.33 ± 0.12
I52A	0.42 ± 0.01
S65A	1.70 ± 0.08
R66A	145.73 ± 10.47
M101A	5.72 ± 0.71

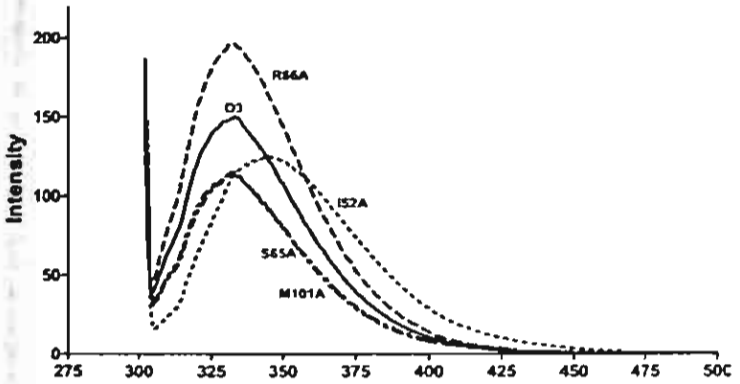


Figure 5 Intrinsic fluorescence spectra of AdGST D3-3 and mutants

Intrinsic fluorescence emission spectra were measured at the excitation wavelength 295 nm, and the λ_{max} and the fluorescence intensity of emission spectra were analysed at a protein concentration of 0.2 mg/ml.

glutathione-binding residues changed the specificity toward various hydrophobic substrates. This suggests a rearrangement of the active site residues, resulting in changes in the topology of the active site pocket. Alternatively, the changes in the G-site residues may result in changes in orientation of glutathione within the active site which then affects the hydrophobic substrate binding. However, R66A showed a decrease in all substrate specificities, which is most likely due to the decreased contribution to GSH ionization, instead of the interaction with the hydrophobic substrates.

Thermal stability

The wild-type enzyme was subjected to a heat inactivation assay, and it was observed that the GST activity began to decrease at 45 °C [46]. This temperature was used to determine half-life stabilities for the recombinant enzymes. The half-life corresponds to the time of incubation when there is 50% remaining activity. The I52A mutant enzyme was shown to be less stable than wild-type by approx. 5-fold (Table 3). However, the replacement at Arg-66 increased the stability of the mutant enzyme by approx. 60-fold. These data demonstrated that Ile-52 and Arg-66 are involved in structural stabilization of the enzyme. The changes in these two residues would appear to change the packing of the active site, which affects the overall structure of the enzyme.

Structural studies on wild-type and mutants

The intrinsic fluorescence spectra show differences between the wild-type, I52A and R66A mutations (Figure 5). Changes in amino acid side chains around tryptophan resulted in an increase

Table 4 Refolding rate constants of adGST D3-3 variants and percentage recovered activity

The enzymes were denatured in 4 M guanidinium chloride in renaturation buffer at 25–27 °C for 30 min and then rapidly diluted (defining time 0) 1:40 into renaturation buffer. Recovered activity was monitored as a function of time by withdrawal of appropriate aliquots of the renaturation mixture and immediately assaying for activity. Refolding rate constants were determined by non-linear regression analysis using a single exponential equation

Enzyme	k_{ref} (min^{-1})	Recovered activity (%)
Wild-type	0.518 ± 0.053	43.8
I52A	25.51 ± 2.53	94
S65A	0.375 ± 0.026	50.3
R66A	0.778 ± 0.249	72.5
M101A	0.279 ± 0.025	36.1

in fluorescence intensity, and a red-shifted spectrum is observed as the protein unfolds to random coil [47]. The λ_{max} values of wild-type and mutant enzymes were the same at 333 ± 1 nm, except for I52A, which had a spectra red shifted to give a λ_{max} at 346 ± 2 nm. These data indicated that the tryptophan residue is more exposed to the solvent, suggesting that the packing of the tertiary structure of the I52A protein is looser. Although the λ_{max} of the R66A enzyme was the same as the wild-type, the intensity of the spectra was increased about 1.6-fold, suggesting that the mutation at this residue causes a rearrangement of the amino acids around the tryptophan that decreased the quenching.

Refolding experiment

The denaturant 4 M guanidinium chloride was sufficient to completely unfold the proteins, as shown by CD spectrum at 222 nm (results not shown). All of the data sets fitted to a single exponential equation for the refolding kinetics. In each case, 5–10 min of incubation in the refolding solution was sufficient to reach the maximum reactivation. In general the reactivation yields of the mutant enzymes were similar to the wild-type (Table 4). However, I52A mutant showed a reactivation velocity about 50 times greater than the wild-type GST, with recovery of about 95% of the activity. This suggests that Ile-52 plays a key role in the folding process.

One possibility may be the side chain of this residue points into the protein molecule and interacts with Leu-6 and Leu-33 to form a small hydrophobic core, as the small side chain of alanine may fit more easily than the side chain of isoleucine for an increased rate in its function in the nucleation of the initial protein folding [36].

CONCLUSION

The present study investigated the glutathione-binding residues of AdGST D3-3 by site-directed mutagenesis. The residues in the G-site appear to impact upon both chemical and physical properties of the enzyme. For example, Ile-52 can affect several aspects of both catalytic and structural properties of the GST. As discussed above the Ile-52 forms a small hydrophobic core with Leu-6 and Leu-33, which, when replaced with the small side chain residue alanine, affected the interaction of the main chain polypeptide between β-sheets 3 and 4, resulting in looser packing of β-sheet 4. Trp-63 is located in β-sheet 4 and the changes at this position have yielded a red shift in intrinsic fluorescence spectra and changes in the half-life and the refolding rate constant of the enzyme. Demonstrating the structural impact of this residue position, Ile-52 is also involved in the initial binding of the glutathione substrate

as shown by GSH affinity changes. Glu-64 had a major effect on structure, because the substitution at this residue caused the enzyme to fold improperly which caused the protein to express as insoluble form. The hydroxy group of Ser-65, which is conserved among the GSTs classes, has a function in glutathione binding and plays an important role in a non-physical step in the enzyme catalysis that is no longer performed by alanine substitution, as demonstrated by the viscosity experiment. Mutational studies of the equivalent amino acid in hGST A1-1 replaced Thr-68 by valine, which caused a shift in pH dependence of the enzyme-catalyzed reaction, suggesting a role in the ionization process of GSH [40]. However, we did not observe the same effect on the mutation in Ser-65 in Delta class which was also seen for Ser-67 in θ class [27]. As shown by the available crystal structure, although Ser-65 is an equivalent residue with the same functional group, the direction of the side chain is different. The hydroxy group of Thr-68 in hGST A1-1 appears to interact with the carboxylate of the glutamate residue of GSH, whereas Ser-65 in adGST D3-3 interacts with the amino group of Arg-66. Arg-66 is involved in the catalytic mechanism through a contribution to the glutathione ionization process, which is why changing this residue changes the rate-limiting step of the enzyme from a physical step to a non-physical step. Although the alignment of GSTs shows variation in this position, several classes of GST still have a conserved arginine residue located in the G-site, for example. Arg-15 in hGST A1-1 [42] and Arg-107 in hGST M2-2 [41]. It has been proposed the positive charge of arginine could provide a counter ion to promote ionization of the sulphydryl group of GSH or assist the function of the glutamate α-carboxylate of GSH in the GSH activation by acting as a proton acceptor in the catalytic mechanism [39–41]. Moreover, this amino acid position also influences structural maintenance of the protein. The R66A mutant enzyme is more stable than the wild-type enzyme, approx. 60-fold, and we also observed a 35 % enhancement in emission intensity which would be a consequence of movements of the indole side chain. The tryptophan may be moving away from the potential quenching effect of Asp-47 and Gln-49 and changing the packing of the active site, as well as affecting the overall structure of the enzyme. The Met-101 position would appear to exert its influence through packing effects. It also may be involved in dimerization, because this residue is located at the subunit interface and interacts with several amino acids from the other subunit. These interaction and packing effect changes then impact upon the enzyme active site as shown by the changes in substrate specificity, as well as changes in k_{cat} and K_m. These effects have been observed previously for a subunit interface residue in a Pi class GST [48].

These experiments demonstrate that glutathione-binding residues contribute to various steps in the catalytic mechanism. Surprisingly, the present studies also implicated the active site residues as contributors to the overall structure with regard to their associations in the folding mechanism and structural stabilization. This contribution was also reflected by the change in the enzyme activity that resulted from an influence on the packing of the protein, in addition to a direct function in the catalytic mechanism.

This work was supported by the Thailand Research Fund and P.W. was supported by a Royal Golden Jubilee Scholarship.

REFERENCES

1 Sheehan, D., Meade, G., Foley, V. M. and Dowd, C. A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem. J. **360**, 1–16

2 Ketterer, B. (2001) A bird's eye view of the glutathione transferase field. Chem. Biol. Interact. **138**, 27–42

3 Armstrong, R. N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem. Res. Toxicol. **10**, 2–18

4 Jakoby, W. B. and Habig, W. H. (1980) In Enzymatic Basis of Detoxication, vol. 2 (Jakoby, W. B., ed.), pp. 63–94, Academic Press, New York

5 Mannervik, B. and Danielson, U. H. (1988) Glutathione transferases – structure and catalytic activity. CRC Crit. Rev. Biochem. **23**, 283–337

6 Hayes, J. D. and Pulford, D. J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol. **30**, 445–600

7 Eaton, D. L. and Bammler, T. K. (1999) Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol. Sci. **49**, 156–164

8 Zhao, T., Singhal, S. S., Piper, J. T., Cheng, J., Pandya, U., Clark-Wronski, J., Awasthi, S. and Awasthi, Y. C. (1999) The role of human glutathione S-transferases hGSTA1-1 and hGSTA2-2 in protection against oxidative stress. Arch. Biochem. Biophys. **367**, 216–224

9 Johansson, A.-S. and Mannervik, B. (2001) Human glutathione transferase A3-3, a highly efficient catalyst of double-bond isomerization in the biosynthetic pathway of steroid hormones. J. Biol. Chem. **276**, 32061–32068

10 Yin, Z., Ivanov, V. N., Habelhah, H., Tew, K. and Ronai, Z. (2000) Glutathione S-transferase p elicits protection against H_2O_2-induced cell death via coordinated regulation of stress kinases. Cancer Res. **60**, 4053–4057

11 Bhargava, M. M., Listowsky, I. and Arias, I. M. (1978) Ligandin. Bilirubin binding and glutathione S-transferase activity are independent processes. J. Biol. Chem. **253**, 4112–4115

12 Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G. and Board, P. (2001) The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. J. Biol. Chem. **276**, 3319–3323

13 Lo Bello, M., Nuccetelli, M., Caccuri, A. M., Stella, L., Parker, M. W., Rossjohn, J., McKinstry, W. J., Mozzi, A. F., Federici, G., Polizio, F. et al. (2001) Human glutathione transferase P1-1 and nitric oxide carriers. A new role for an old enzyme. J. Biol. Chem. **276**, 42138–42145

14 Dirr, H., Reinemer, P. and Huber, R. (1994) X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. Eur. J. Biochem. **220**, 645–661

15 Board, P. G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L. S., Schulte, G. K., Danley, D. E., Hoth, L. R., Griffor, M. C., Kamath, A. V. et al. (2000) Identification, characterization, and crystal structure of the Omega class glutathione transferases. J. Biol. Chem. **275**, 24798–24806

16 Ji, X., Von Rosenvinge, E. C., Johnson, W. W., Tomarev, S. I., Piatigorsky, J., Armstrong, R. N. and Gilliland, G. L. (1995) Three-dimensional structure, catalytic properties, and evolution of a sigma class glutathione transferase from squid, a progenitor of the lens S-crystallins of cephalopods. Biochemistry **34**, 5317–5328

17 Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G. and Parker, M. W. (1992) Three-dimensional structure of class π glutathione S-transferase from human placenta in complex with S-hexylglutathione at 2.8 Å resolution. J. Mol. Biol. **227**, 214–226

18 Reinemer, P., Prade, L., Hof, P., Neuefeind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Bartunik, H. D. and Bieseler, B. (1996) Three-dimensional structure of glutathione S-transferase from *Arabidopsis thaliana* at 2.2 Å resolution: structural characterization of herbicide-conjugating plant glutathione S-transferases and a novel active site architecture. J. Mol. Biol. **255**, 289–309

19 Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., [illegible] G. L., Armstrong, R. N., Ji, X., Board, P. G. et al. (1993) Structure determination and refinement of human Alpha class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. J. Mol. Biol. **232**, 192–212

20 Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W. (1995) Crystal structure of a theta class glutathione transferase. EMBO J. **14**, 2133–2143

21 Wilce, M. C. J. and Parker, M. W. (1994) Structure and function of glutathione S-transferases. Biochim. Biophys. Acta **1205**, 1–18

22 Armstrong, R. N., Rife, C. and Wang, Z. (2001) Structure, mechanism and evolution of thiol transferases. Chem. Biol. Interact. **133**, 167–169

23 Caccuri, A. M., Ascenzi, P., Antonini, G., Parker, M. W., Oakley, A. J., Chiessi, E., Nuccetelli, M., Battistoni, A., Bellizia, A. and Ricci, G. (1996) Structural flexibility modulates the activity of human glutathione transferase P1-1. Influence of a poor co-substrate on dynamics and kinetics of human glutathione transferase. J. Biol. Chem. **271**, 16193–16198

24 Caccuri, A. M., Antonini, G., Nicotra, M., Battistoni, A., Lo Bello, M., Board, P. G., Parker, M. W. and Ricci, G. (1997) Catalytic mechanism and role of hydroxyl residues in the active site of theta class glutathione S-transferases. Investigation of Ser-9 and Tyr-113 in a glutathione S-transferase from the Australian sheep blowfly, *Lucilia cuprina*. J. Biol. Chem. **272**, 29681–29686

25 Caccuri, A. M., Lo Bello, M., Nuccetelli, M., Rossi, P., Antonini, G., Federici, G. and Ricci, G. (1998) Proton release upon glutathione binding to glutathione transferase P1-1 kinetic analysis of a multistep glutathione binding process. Biochemistry **37**, 3028–3034
26 Lo Bello, M., Battistoni, A., Mazzetti, A. P., Board, P. G., Muramatsu, M., Federici, G. and Ricci, G. (1995) Site-directed mutatgenesis of human glutathione transferase P1-1 Spectral, kinetic, and structural properties of Cys-47 and Lys-54 mutants. J. Biol. Chem. **270**, 1249–1253
27 Labrou, N. E., Mello, L. V. and Clonis, Y. D. (2001) Functional and structural roles of the glutathione-binding residues in maize (*Zea mays*) glutathione S-transferase I. Biochem. J. **358**, 101–110
28 Jirajaroenrat, K., Pongjaroenkit, S., Kruttanai, C., Prapanthadara, L. and Ketterman, A. J. (2001) Heterologous expression and characterization of alternatively spliced glutathione *S*-transferases from a single *Anopheles* gene. Insect Biochem. Mol. Biol. **31**, 867–875
29 Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L. and Ketterman, A. J. (2001) Genomic organization and putative promotors of highly conserved glutathione S-transferases originating by alternative splicing in *Anopheles dirus*. Insect Biochem. Mol. Biol. **31**, 75–85
30 Oakley, A. J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A. J. and Wilce, M. C. J. (2001) The crystal structures of glutathione S-transferases isozymes 1-3 and 1-4 from *Anopheles dirus* species B. Prot. Sci. **10**, 2176–2185
31 Wongsantichon, J., Harnnoi, T. and Ketterman, A. J. (2003) A sensitive core region in the structure of glutathione S-transferases. Biochem. J. **373**, 759–765
32 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
33 Caccuri, A. M., Ascenzi, P., Lo Bello, M., Federici, G., Battistoni, A., Mazzetti, P. and Ricci, G. (1994) Are the steady state kinetics of glutathione transferase always dependent on the deprotonation of the bound glutathione? New insights in the kinetic mechanism of GST P1-1. Biochem. Biophys. Res. Commun. **200**, 1428–1434
34 Wolf, A. V., Brown, M. G. and Prentiss, P. G. (1985) Concentrative properties of aqueous solutions: conversion tables. In Handbook of Chemistry and Physics, pp. D219–D232, CRC Press, Inc., Boca Raton, Florida
35 Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. **249**, 7130–7139
36 Vararattanavech, A. and Ketterman, A. (2003) Multiple roles of glutathione binding-site residues of glutathione S-transferase. Protein Pept. Lett. **10**, 441–448
37 Stenberg, G., Dragani, B., Cocco, R., Mannervik, B. and Aceto, A. (2000) A conserved 'hydrophobic staple motif' plays a crucial role in the refolding of human glutathione transferase P1-1. J. Biol. Chem. **275**, 10421–10428
38 Caccuri, A. M., Antonini, G., Board, P. G., Parker, M. W., Nicotra, M., Lo Bello, M., Federici, G. and Ricci, G. (1999) Proton release on binding of glutathione to Alpha, Mu and Delta class glutathione transferases. Biochem. J. **344**, 419–425
39 Gustafsson, A., Pettersson, P. L., Grehn, L., Jemth, P. and Mannervik, B. (2001) Role of the glutamyl α-carboxylate of the substrate glutathione in the catalytic mechanism of human glutathione transferase A1-1. Biochemistry **40**, 15835–15845
40 Widersten, M., Björnestedt, R. and Mannervik, B. (1996) Involvement of the carboxyl groups of glutathione in the catalytic mechanism of human glutathione transferase A1-1. Biochemistry **35**, 7731–7742
41 Patskovsky, Y. V., Patskovska, L. N. and Listowsky, I. (2000) The enhanced affinity for thiolate anion and activation of enzyme-bound glutathione is governed by an arginine residue of human Mu class glutathione S-transferases. J. Biol. Chem. **275**, 3296–3304
42 Björnestedt, R., Stenberg, G., Widersten, M., Board, P. G., Sinning, I., Jones, T. A. and Mannervik, B. (1995) Functional significance of arginine 15 in the active site of human class alpha glutathione transferase A1-1. J. Mol. Biol. **247**, 765–773
43 Johnson, W. W., Liu, S., Ji, X., Gilliland, G. L. and Armstrong, R. N. (1993) Tyrosine 115 participates both in chemical and physical steps of the catalytic mechanism of a glutathione S-transferase. J. Biol. Chem. **268**, 11508–11511
44 Jemth, P. and Mannervik, B. (1999) Fast product formation and slow product release are important features in a hysteretic reaction mechanism of glutathione transferase T2-2. Biochemistry **38**, 9982–9991
45 Allardyce, C. S., McDonagh, P. D., Lian, L.-Y., Wolf, C. R. and Roberts, G. C. K. (1999) The role of tyrosine-9 and the C-terminal helix in the catalytic mechanism of Alpha-class glutathione S-transferases. Biochem. J. **343**, 525–531
46 Wongtrakul, J., Udomsinprasert, R. and Ketterman, A. (2003) Non-active site residues Cys69 and Asp150 affected the enzymatic properties of glutathione S-transferase AdGSTD3-3. Insect Biochem. Mol. Biol. **33**, 971–979
47 Dirr, H. W. and Wallace, L. A. (1999) Role of the C-terminal helix 9 in the stability and ligandin function of class a glutathione transferase A1-1. Biochemistry **38**, 15631–15640
48 Stenberg, G., Abdalla, A.-M. and Mannervik, B. (2000) Tyrosine 50 at the subunit interface of dimeric human glutathione transferase P1-1 is a structural key residue for modulating protein stability and catalytic function. Biochem. Biophys. Res. Commun. **271**, 59–63

Received 28 April 2004/1 June 2004; accepted 8 June 2004
Published as BJ Immediate Publication 8 June 2004, DOI 10.1042/BJ20040697

Biochem. J. (2003) 373, 759–765 (Printed in Great Britain)

A sensitive core region in the structure of glutathione S-transferases

Jantana WONGSANTICHON, Thasaneeya HARNNOI and Albert J. KETTERMAN[1]

Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand

A variant form of an *Anopheles dirus* glutathione S-transferase (GST), designated AdGSTD4-4, possesses a single amino acid change of leucine to arginine (Leu-103-Arg). Although residue 103 is outside of the active site, it has major effects on enzymic properties. To investigate these structural effects, site-directed mutagenesis was used to generate mutants by changing the non-polar leucine to alanine, glutamate, isoleucine, methionine, asparagine, or tyrosine. All of the recombinant GSTs showed approximately the same expression level at 25 °C. Several of the mutants lacked glutathione (GSH)-binding affinity but were purified by S-hexyl-GSH-based affinity chromatography. However the protein yields (70-fold lower), as well as the GST activity (100-fold lower), of Leu-103-Tyr and Leu-103-Arg purifications were surprisingly low and precluded the performance of kinetic experiments. Size-exclusion chromatography showed that both GSTs Leu-103-Tyr and Leu-103-Arg formed dimers. Using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH substrates to determine kinetic constants it was demonstrated that the other Leu-103 mutants possessed a greater K_m towards GSH and a differing K_m towards CDNB. The V_{max} ranged from 44.7 to 87.0 μmol/min per mg (wild-type, 44.7 μmol/min per mg). Substrate-specificity studies showed different selectivity properties for each mutant. The structural residue Leu-103 affects the active site through H-bond and van-der-Waal contacts with six active-site residues in the GSH binding site. Changes in this interior core residue appear to disrupt internal packing, which affects active-site residues as well as residues at the subunit–subunit interface. Finally, the data suggest that Leu-103 is noteworthy as a sensitive residue in the GST structure that modulates enzyme activity as well as stability.

Key words: *Anopheles dirus*, non-active site, structure–function.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are well known as dimeric detoxification enzymes that catalyse a wide variety of conjugations of glutathione (GSH) to hydrophobic toxic compounds. Each subunit of 25 kDa contains a catalytic pocket of the GSH binding site (G-site) and the nearby hydrophobic substrate-binding site (H-site) [1–3]. In general, the N-terminal domain of GST enzymes comprises most of the G-site and is quite conserved across classes, whereas the C-terminal domain that forms part of the H-site is more divergent. Variations in hydrophobic residues of the H-site, between different GSTs, are proposed to play a role in substrate selectivity.

Previously we have reported *adgst1AS1* as an *Anopheles dirus* alternatively spliced GST gene [4]. This gene encodes four Delta class (insect class 1) GST enzymes: adGST1-1, adGST1-2, adGST1-3 and adGST1-4, which share 61–77 % amino acid identity [5]. The four splicing products were named according to the insect GST nomenclature in use. However, to be in alignment with a proposed universal GST nomenclature, we have renamed the enzymes AdGSTD1-1, AdGSTD2-2, AdGSTD3-3 and AdGSTD4-4 [6]. The 'D' refers to the GST Delta class and the subunit number remains the same since the subunits were enumerated as they were initially discovered. The double number, GSTD3-3 for example, signifies that the enzyme is a homodimer. These four GSTs share an untranslated exon 1 and a translated exon 2 that codes for 45 amino acids at the N-terminus. These two exons are spliced to one of four alternative versions of exon 3 (3A, 3B, 3C or 3D) to generate four different mature transcripts. A homologous GST gene has also been reported in *Anopheles gambiae* as *aggst1α* [7]. The *A. dirus* and *A. gambiae* genes have a conserved exon arrangement with approximately 79 % nucleotide identity. The four GST proteins from each of the two mosquito species have 85–92 % amino acid identity between the homologous enzymes. These two mosquito species are both malaria vectors, although they are geographically distant, with *A. dirus* in Southeast Asia and *A. gambiae* in Africa. The two Anopheline species must have diverged several million years ago; therefore the high conservation and type of gene organization suggests this gene is very important. In fact, the four splice products are active GSTs possessing distinct enzyme-kinetic properties. The insecticide-interaction properties, the available crystal structure for AdGSTD4-4, and the canonical nature of GST tertiary structure make AdGSTD4-4 an ideal candidate for further structure–function studies [5,8].

As well as allelic variants (i.e. variants from a single gene locus), it has been shown that site-directed mutagenesis variants with single residue changes could influence the kinetic property, thermal stability, substrate affinity, substrate specificity, and/or catalytic activity of a GST enzyme [9–14]. Studies of allelic variants provide useful information for elucidation of structure–function relationships. Several variant forms of AdGSTD4-4, obtained by reverse transcriptase PCR, yielded amino acid residue changes distributed throughout the tertiary structure. One of these proteins, GST Leu-103-Arg, markedly differed from the other GSTs. This substitution, at position 103, was of particular interest since the change from the non-polar leucine to a positively charged arginine reduced most of the enzymic activity. In addition, this protein could not be purified using GSH-based affinity chromatography. An available X-ray crystal structure showed that the 103

Abbreviations used: GST, glutathione S-transferase; GSH, glutathione; G-site, glutathione binding site; H-site, hydrophobic substrate-binding site; CDNB, 1-chloro-2,4-dinitrobenzene; Leu-103-Arg, denotes substitution of one amino acid residue with another, e.g. Leu-103 with Arg-103.

[1] To whom correspondence should be addressed (e-mail frakt@mahidol.ac.th).

sition does not form part of the active site pocket of the enzyme, is making this residue of interest for further studies [8].

To elucidate the importance of this residue position, site-ected mutagenesis was employed to change the leucine to other residues: alanine, glutamate, isoleucine, methionine, paragine, or tyrosine, respectively. The mutant isoform types herated for the Leu-103 residue included: hydrophobic, sitively charged, negatively charged, polar uncharged, as well as ose possessing various sizes and/or lengths of functional group. gether with available tertiary structure [8,15], enzyme kinetic operties were investigated to provide a clearer understanding of e structure–function relationship.

XPERIMENTAL PROCEDURES

xpression and purification of recombinant AdGSTD4-4 protein

Cloning and heterologous expression of the *A. dirus* GSTs were erformed as described previously [5]. The mutant construction nethod was based on the Stratagene Quick Change™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The oligonucleotide primers were designed to introduce a single esidue mutation into AdGSTD4-4 to change leucine at the 103 osition to alanine, glutamate, isoleucine, methionine, asparagine or tyrosine. In the primers shown below, altered nucleotides are shown in bold and additional recognition sites of restriction endonucleases (*Sac*II for the Leu-103-Ala and Leu-103-Ile sets; *Eco*RI for the Leu-103-Glu set; *Hin*cII for the Leu-103-Met and Leu-103-Tyr sets; *Xmn*I for the Leu-103-Asn set) are underlined: Leu-103-Ala reverse, 5′-CAC ATC GAA GAA CGC CCG CTG GTG GAC GAC GGC ACG GCG CCG CGG ATC GCT TG-3′; Leu-103-Ala forward, 5′-CAA GCG ATC CGC GGC GCC GTG CCG TCG TCC ACC AGC GGG CGT TCT TCG ATG TG-3′; Leu-103-Glu reverse, 5′-CAC ATC GAA GAA TTC CCG CTG GTG GAC-3′; Leu-103-Glu forward, 5′-GTC CAC CAG CGG GAA TTC TTC GAT GTG-3′; Leu-103-Ile reverse, 5′-CAC ATC GAA GAA GAT CCG CTG GTG GAC GAC GGC ACG GCG CCG CGG ATC GCT TG-3′; Leu-103-Ile forward, 5′-CAA GCG ATC CGC GGC GCC GTG CCG TCG TCC ACC AGC GGA TCT TCT TCG ATG TG-3′; Leu-103-Met reverse, 5′-GAA GAA CAT CCG CTG GTG GAC GAC GGC ACG TCG AC-3′; Leu-103-Met forward, 5′-GTC GAC GTG CCG TCG TCC ACC AGC GGA TGT TCT TC-3′; Leu-103-Asn reverse, 5′-CCA CCA GCG GAA CTT CTT CGA TGT GGC C-3′; Leu-103-Asn forward, 5′-GGC CAC ATC GAA GAA GTT CCG CTG GTG G-3′; Leu-103-Tyr reverse, 5′-GAA GAA ATA CCG CTG GTG GAC GAC GGC ACG TCG AC-3′; Leu-103-Tyr forward, 5′-GTC GAC GTG CCG TCG TCC ACC AGC GGT ATT TCT TC-3′. The expression construct of AdGSTD4-4 was used as template DNA. The soluble recombinant GST proteins were purified by GSTrap affinity chromatography (Amersham Biosciences, Piscataway, NJ, U.S.A.) or S-hexylglutathione agarose (Sigma) affinity chromatography, in the case of low affinity towards the GSH ligand [5,9].

Enzyme characterization and protein assay

The substrate-specificity studies and steady-state kinetics were performed as described previously [5,16,17]. The data shown are means ± standard deviations from at least three independent experiments. Protein concentration was determined by the Bradford method [18] using the Bio-Rad Laboratories (Hercules, CA, U.S.A.) protein reagent with bovine serum albumin as standard protein.

Size-exclusion chromatography

An *Escherichia coli* lysate of expressed enzymes was filtered through a 0.45 micron Acrodisc® syringe filter (Pall Life Sciences, Sydney, Australia) and fractionated through a Superdex 75 HR 10/30 column (Amersham Biosciences) at a flow rate of 0.5 ml/min with a pressure of 1 MPa on an ÄKTA™ purifier liquid chromatography system (Amersham Biosciences). Chymotrypsinogen A (25 kDa ± 25 %) and ovalbumin (43 kDa ± 15 %) were used as molecular mass standards. The buffer used contained 50 mM K_2HPO_4/KH_2PO_4 and 150 mM NaCl, pH 7.0. The elution was collected as 1 ml fractions.

Heat inactivation assay

Enzymes derived from the different isoforms, each at a concentration of 0.05 mg/ml [in 0.1 M phosphate buffer (pH 6.5) containing 5 mM DTT and 1 mM EDTA], were incubated at various temperatures for 10 min. The temperatures used were between 25 °C to 60 °C in 5 °C increments. An appropriate amount of incubated mixture was taken to assay for remaining GST activity [19].

Half-life determination

Enzymes derived from the different isoforms [each at a concentration of 0.05 mg/ml in 0.1 M phosphate buffer (pH 6.5) containing 5 mM DTT and 1 mM EDTA] were incubated at 45 °C. An appropriate amount of incubated mixture (adjusted for the specific activity of each mutant enzyme, e.g. an amount of approx. 10 μl of incubated mixture was used for the mutant GSTs in this study) was taken to assay for remaining GST activity at various time points ranging from 0 to 15 minutes. Log percentage of original activity was plotted versus pre-incubation times. Slopes from linear regression analysis using GraphPad Prism 2.01 software were employed in the half-life calculation.

RESULTS AND DISCUSSION

Recombinant protein expression and purification

All mutant isoforms were successfully generated by site-directed mutagenesis. DNA sequencing was used to confirm each single nucleotide at least twice. Protein expression at 37 °C showed that Leu-103-Asn and Leu-103-Tyr were expressed as insoluble inclusion bodies within the *E. coli* cells and this created difficulties in the purification process. The temperature of induction was lowered to 25 °C and then all mutant isoforms were expressed as soluble proteins of approximately the same amount (Figure 1). Temperature-sensitive GST mutants have been observed previously, where the mutations impact upon important structural motifs [19–23]. The temperature-sensitive folding mutants were obtained when expressed at a permissive temperature such as 25 °C. It was suggested that these mutants fail to attain the native conformation at higher temperatures, such as 37 °C, and are therefore expressed in lower amounts or as insoluble inclusion bodies. The expression of two mutant (at residue 103) proteins as inclusion bodies suggests that these residue changes impact significantly upon the structure during the initial folding process.

GST activity was also undetectable in the lysates from Leu-103-Arg and Leu-103-Tyr mutants, therefore these lysates were subjected to size-exclusion chromatography to investigate whether these mutants were expressed in an active dimeric, or inactive monomeric, form. The result showed that the GST was

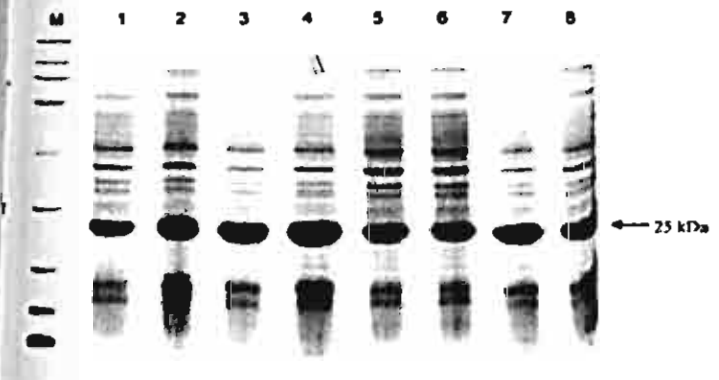


Figure 1 Protein expression of Leu-103 mutant isoforms

An amount of *E. coli* lysate (adjusted to give a D_{600} of 0.1 for each isoform) was resuspended in 16 μl of loading buffer and separated by SDS/PAGE. Lane 1, Leu-103-Arg; lane 2, Leu-103-Tyr; lane 3, Leu-103-Asn; lane 4, Leu-103-Met; lane 5, Leu-103-Ile; lane 6, Leu-103-Glu; lane 7, Leu-103-Ala; and lane 8, wild-type, respectively. M, broad-range molecular mass marker.

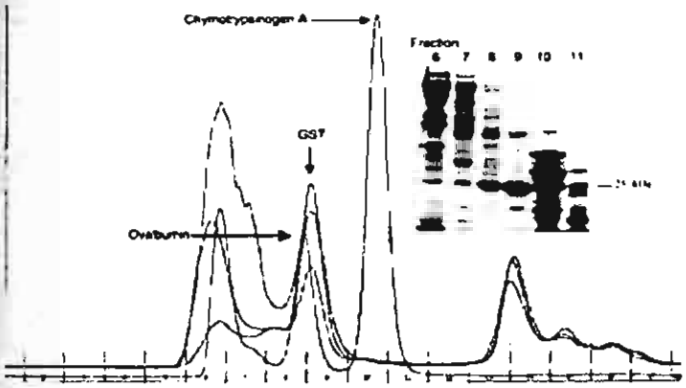


Figure 2 FPLC chromatogram of AdGSTD4-4 (wild-type), Leu-103-Arg, and Leu-103-Tyr

Ovalbumin (43 kDa ± 15 %) and chymotrypsinogen A (25 kDa ± 25 %) were used as molecular mass standard markers for the dimeric and monomeric form, respectively. A polyacrylamide gel (inset) shows relative sizes of fractionated proteins.

observed at a point corresponding to a dimeric molecular mass of 50 kDa (Figure 2). We then concluded that the Leu-103-Arg and Leu-103-Tyr mutants were expressed as homodimers.

Not all recombinant GSTs were effectively purified by the GSTrap column. The expressed enzymes of Leu-103-Glu, Leu-103-Asn, Leu-103-Arg, and Leu-103-Tyr, failed to bind to the GSTrap column but were successfully purified by using an S-hexylglutathione affinity column. Nevertheless, the purification yield of Leu-103-Arg and Leu-103-Tyr was low, approx. 1% of the wild-type yield (Table 1). Differences in the purification yield for each recombinant protein may have resulted from a reduction in binding affinity to the ligand on the gel matrix of either the GSTrap column or the S-hexylglutathione agarose. However, all the recombinant enzymes retained the ability to bind to the GSH substrate, albeit with different affinities compared with wild-type, as indicated by the K_m values. A point worth noting is that any amino acid substitution, with a non-hydrophobic residue in the 103 position, would lose the binding affinity to the GSTrap, a GSH-based affinity matrix. The X-ray crystal structure of AdGSTD4-4 shows that Leu-103 is not in the active site but is located in helix 4 and is in a hydrophobic environment (Figure 3). It appears that the alteration of this position to a non-hydrophobic residue disrupts the conformation of, and decreases the ability to bind to, the GSH-based affinity matrix. Changes in GST interaction with GSH-based affinity matrices has been reported in studies with an Alpha and a Pi class GSTs involving a single amino acid important in intersubunit interactions [24,25]. This residue (Phe-51 in Alpha and Tyr-50 in Pi class) was involved in a structural lock-and-key motif contributing to the subunit–subunit interface. Although a structural residue, this residue position impacted significantly on enzyme activity such that the mutant proteins displayed a decreased affinity for the GSH affinity matrices. This residue position is located between α helix 2 and β strand 3, and parts of this region contribute to one side of the G-site [25]. In our study, residue 103 is not equivalent to the aromatic residue in Alpha and Pi class GSTs, but is located in the interior of the subunit, also adjacent to the active site and the subunit interface.

Enzyme kinetic properties

The steady-state kinetics followed Michaelis–Menten kinetics for several of the mutants and the kinetic parameters were determined by non-linear regression analysis and compared with the wild-type AdGSTD4-4 (Table 1). Changes of the Leu-103 residue to arginine or tyrosine almost abolished GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB), which precluded the determination of kinetic parameters for these two mutants. However, all other mutant enzymes had a greater maximum rate of reaction or V_{max} (Table 1). The catalytic efficiencies or k_{cat}/K_m of all mutant enzymes towards CDNB were 1.2–3-fold greater than the wild-type enzyme. But the catalytic efficiencies towards GSH of the Leu-103 mutants were 0.02–0.37-fold less than the

Table 1 Yields of purification and kinetic parameters of the seven recombinant mutant GSTs compared with AdGSTD4-4 (wild-type) from *A. dirus*

The data are the means ± S.D. of at least three separate experiments. n.d., not determined.

Clone	Protein yield (mg/l)	V_{max} (μmol/min per mg)	k_{cat} (s^{-1})	CDNB		GSH	
				K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Wild-type	43.2	44.7 ± 2.3	18.60	0.76 ± 0.07	24.5	0.70 ± 0.09	26.6
Leu-103-Ala	8.0	47.6 ± 3.6	19.78	0.62 ± 0.19	31.9	24.0 ± 2.40	0.8
Leu-103-Glu	4.8	76.6 ± 2.7	31.90	0.72 ± 0.07	44.3	3.42 ± 0.27	9.3
Leu-103-Ile	26.6	87.0 ± 1.4	36.21	0.49 ± 0.08	73.9	3.70 ± 0.37	9.8
Leu-103-Met	70.0	72.4 ± 5.2	30.16	0.91 ± 0.14	33.1	6.38 ± 0.55	4.7
Leu-103-Asn	20.0	52.7 ± 3.4	21.94	0.77 ± 0.09	28.5	37.4 ± 2.66	0.6
Leu-103-Tyr	0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Leu-103-Arg	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

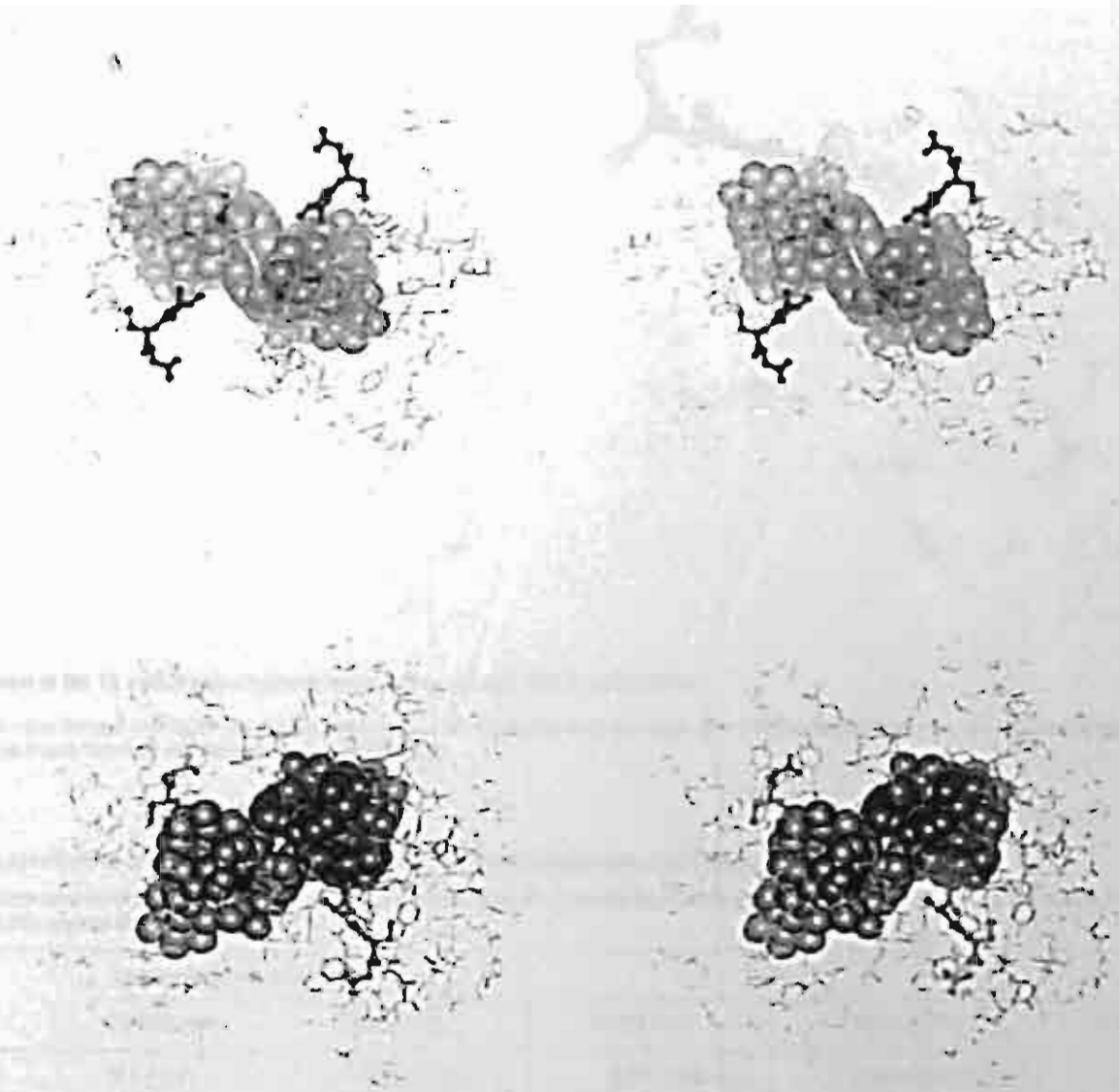


Figure 3 Stereo view of the 13 amino acid residues surrounding residue 103 in AdGSTD4-4

The upper panel is looking down on to the active site, with GSH superimposed as a green ball-and-stick figure. The lower panel shows the structure rotated by 180°. The two subunits are shown in grey and gold and L103 is shown in blue. The coordinates for the tertiary structure have been deposited in the Protein Data Bank with the accession number 1JLW [8].

wild-type enzyme. Except for Leu-103-Arg and Leu-103-Tyr, it appears that all other Leu-103 mutants possess a greater K_mGSH (K_m towards GSH substrate), ranging from 3.4–37.4 mM (wild-type, 0.7 mM), indicating a decreased affinity of these mutant enzymes for the GSH substrate. Several of the Leu-103 mutants, most notably Leu-103-Ala and Leu-103-Asn, instead of a hyperbola, yielded a sigmoidal curve-shape for GSH binding. This was also reported for several Tyr-50 mutants in GSTP1-1 which was thought to impact upon part of the G-site [25]. A sigmoidal velocity curve reflects the GSH binding in the first active site, which then facilitates another GSH binding in the second active site by increasing the binding affinity of the vacant binding site [26]. The X-ray crystal structure of AdGSTD4-4 shows that the Leu-103 residue is not in a position to be involved in main-chain or side-chain interactions with GSH in the active site. However, there are 13 residues packed in a sphere around Leu-103 involving 34 H-bonds with contacts extending across the subunits (Figure 4). These 13 residues are Arg-67, Ile-71, Tyr-89, Val-99, His-100, Phe-104, Phe-104B (the other subunit), Asp-106, Val-107, Ile-163, Ala-164, Ser-167 and Ile-168. Six of these residues are in the active site: Arg-67, Phe-104, Phe-104B, Asp-106, Val-107 and Ser-167, and interact with an additional five active site residues: Glu-65, Ala-108, Ala-108B (the other subunit), Tyr-111 and Thr-171. Residue Arg-67, which is a strictly conserved residue through the GST classes, directly interacts with the carboxylic acid of the γ-glutamyl of GSH. The comparative arginine residue, Arg-69 in human alpha class GST, has been shown to be important for GSH binding as well as stabilization of the enzyme conformation [27]. The variation in K_mCDNB may originate from charge distribution effects transmitted between the G-site and H-site. This has previously been suggested for GSTs where the electrostatic field of the active site had been modified or disturbed by residue changes [28–30]. A corollary to changes in the electrostatic field of the active site would be changes in the topology of the active site through residue movement, both through adjustments in packing and conformational flexing differences.

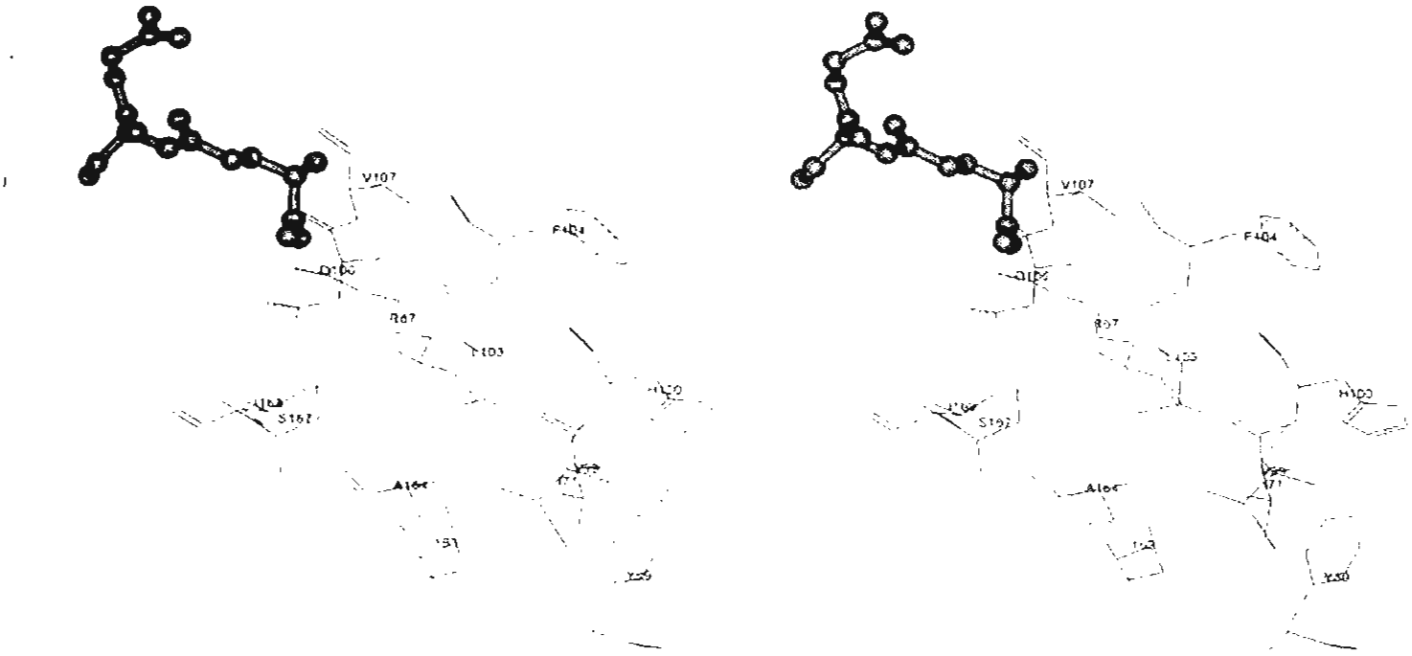


Figure 4 Stereo view of the 13 amino acid residues surrounding residue 103 in AdGSTD4-4

GSH is superimposed as a grey ball-and-stick figure. The residues from subunit A are represented in black lines and Phe-104 from subunit B as grey lines. The co-ordinates for the tertiary structure have been deposited in the Protein Data Bank with the accession number 1JLW [8].

Table 2 Substrate specificities of the seven recombinant mutant GSTs compared with AdGSTD4-4 (wild-type) from *A. dirus*

The substrate concentrations used are in parentheses. DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; PNPB, p-nitrophenethyl bromide; PNBC, p-nitrobenzyl chloride. The data are the means ± S.D. of at least three separate experiments.

	Substrate specificity (μmol/min per mg)				
Clone	CDNB (3 mM)	DCNB (1 mM)	EA (0.2 mM)	PNPB (0.1 mM)	PNBC (0.1 mM)
Wild-type	37.4 ± 2.2	0.016 ± 0.003	0.211 ± 0.019	0.042 ± 0.008	0.060 ± 0.005
Leu-103-Ala	19.7 ± 1.8	0.017 ± 0.009	0.313 ± 0.018	< 0.091	< 0.057
Leu-103-Glu	46.4 ± 0.4	0.032 ± 0.005	0.345 ± 0.050	< 0.078	0.102 ± 0.031
Leu-103-Ile	50.4 ± 4.7	0.046 ± 0.004	0.124 ± 0.020	0.030 ± 0.004	0.058 ± 0.012
Leu-103-Met	38.6 ± 1.9	0.039 ± 0.007	0.182 ± 0.012	0.027 ± 0.002	0.056 ± 0.002
Leu-103-Asn	15.5 ± 0.8	0.009 ± 0.001	< 0.004	< 0.018	0.030 ± 0.004
Leu-103-Tyr	0.66 ± 0.11	< 0.071	< 0.121	< 0.503	< 0.318
Leu-103-Arg	0.36 ± 0.02	< 0.066	< 0.113	< 0.470	< 0.297

Substrate specificities

Substrate specificity determination revealed differences in the specificity or the interaction of the mutant enzymes with several hydrophobic substrates, which indicated that the changed single amino acid residues affected the catalytic specificity of the enzymes (Table 2). It has been shown in human Pi GST (Pi class GST) that there are sensitive structural regions where single residue changes influence inhibitor specificity and decrease enzyme activity [27,31]. Some of these residues are found in the domain 2 α-helices that generate a hydrophobic core. This hydrophobic core is thought to stabilize the subunit tertiary structure. Previously we have shown for AdGSTD1-1 that residues outside the active site can subtly affect the enzyme specificity [9]. In AdGSTD1-1, one of these residues was found on α helix 7 that contributes to the hydrophobic core in domain 2. The differing conformations may bring different residues into proximity or change the proximity/orientation of the residues involved in binding or catalysis, thereby affecting the observed enzyme properties. This has also been shown by mutagenesis studies of human Theta class GSTT2-2 [32].

Enzyme stability

The wild-type enzyme was subjected to a heat-inactivation assay and it was observed that the GST activity began to decrease at 45 °C. This temperature was used to determine half-life stabilities for recombinant enzymes. The half-life corresponds to the time of incubation at which there is 50 % of activity remaining. Most of

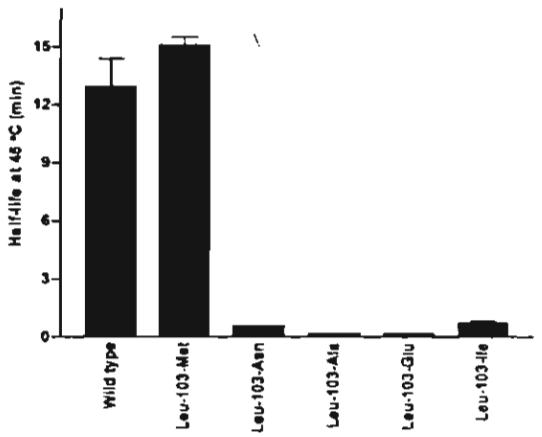


Figure 5 Half-life of recombinant mutant GSTs at 45 °C compared with rdGSTD4-4 (wild-type) from *A. dirus*

The data are the means ± S.D. of at least three separate experiments.

the Leu-103 mutants possess a lower stability, although Leu-103-Met showed a slight increase in stability (Figure 5). It has been shown previously that the mutation of a single amino acid residue can have an impact on stability of the enzyme [24,25]. These studies of Alpha and Pi GSTs characterized a residue at the subunit interface involved in the dimerization of the subunits. The present study characterizes a residue, Leu-103, which is in the interior of the subunit. This position, with its surrounding 13 residues, forms a hydrophobic core completely excluding water molecules in the centre of the subunit. The mutations of residue 103 disturb the packing of these residues, which affects the stability of the structure as well as its enzymic properties.

Conclusion

It has been shown that the structural residue Leu-103 affects the active site through H-bond and van-der-Waal contacts with six active-site residues in the G-site. Changes in this interior core residue appear to disrupt internal packing, affecting active-site residues as well as residues at the subunit–subunit interface. These effects are observed as changes in k_{cat} and K_m for the hydrophobic substrate CDNB as well as changes in K_m for GSH. The Leu-103 effects also influence substrate specificity as well as enzyme stability.

This work was funded by the Thailand Research Fund (TRF)

REFERENCES

1 Mannervik, B. and Danielson, U. H. (1988) Glutathione transferases – structure and catalytic activity. CRC Crit. Rev. Biochem. **23**, 283–337
2 Wilce, M. C. J. and Parker, M. W. (1994) Structure and function of glutathione S-transferases. Biochim. Biophys. Acta **1205**, 1–18
3 Armstrong, R. N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem. Res. Toxicol. **10**, 2–18
4 Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L. and Ketterman, A. J. (2001) Genomic organization and putative promotors of highly conserved glutathione S-transferases originating by alternative splicing in *Anopheles dirus*. Insect Biochem. Molec. Biol. **31**, 75–85
5 Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L. and Ketterman, A. J. (2001) Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single *Anopheles* gene. Insect Biochem. Molec. Biol. **31**, 867–875
6 Chelvanayagam, G., Parker, M. W. and Board, P. G. (2001) Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. Chemico-Biological Interactions **133**, 256–260
7 Ranson, H., Collins, F. and Hemingway, J. (1998) The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione S-transferase family. Proc. Natl. Acad. Sci. U.S.A. **95**, 14284–14289
8 Oakley, A. J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A. J. and Wilce, M. C. J. (2001) The crystal structures of glutathione S-transferases isozymes 1-3 and 1-4 from *Anopheles dirus* species B. Protein Science **10**, 2176–2185
9 Ketterman, A. J., Prommeenate, P., Boonchauy, C., Chanama, U., Leetachewa, S., Promtet, N. and Prapanthadara, L. (2001) Single amino acid changes outside the active site significantly affect activity of glutathione S-transferases. Insect Biochem. Molec. Biol. **31**, 65–74
10 Johansson, A.-S., Stenberg, G., Widersten, M. and Mannervik, B. (1998) Structure–activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. J. Mol. Biol. **278**, 687–698
11 Xia, H., Gu, Y., Pan, S. S., Ji, X. and Singh, S. V. (2001) Amino acid substitutions at positions 207 and 221 contribute to catalytic differences between murine glutathione S-transferase A1-1 and A2-2 toward (+)-anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Biochemistry **38**, 19824–19830
12 Chern, M.-K., Wu, T.-C., Hsieh, C.-H., Chou, C.-C., Liu, L.-F., Kuan, I.-C., Yeh, Y.-H., Hsiao, C.-D. and Tam, M. F. (2000) Tyr115, Gln165 and Trp209 contribute to the 1,2-epoxy-3-(p-nitrophenoxy)propane-conjugating activity of glutathione *S*-transferase cGSTM1. J. Mol. Biol. **300**, 1257–1269
13 Gustafsson, A., Etahadieh, M., Jemth, P. and Mannervik, B. (1999) The C-terminal region of human glutathione transferase A1-A1 affects the rate of glutathione binding and the ionization of the active-site Tyr9. Biochemistry **38**, 16268–16275
14 Allocati, N., Casalone, E., Massuli, M., Polekhina, G., Rossjohn, J., Parker, M. W. and Di Ilio, C. (2000) Evaluation of the role of two conserved active-site residues in Beta class glutathione S-transferases. Biochem. J. **351**, 341–346
15 Oakley, A. J., Jirajaroenrat, K., Harnnoi, T., Ketterman, A. J. and Wilce, M. C. J. (2001) Crystallization of two glutathione S-transferases from an unusual gene family. Acta Crystallogr. Sect. D Biol. Crystallogr. **57**, 870–872
16 Ranson, H., Prapanthadara, L. and Hemingway, J. (1997) Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of *Anopheles gambiae*. Biochem. J. **324**, 97–102
17 Prapanthadara, L., Koottathep, S., Promtet, N., Hemingway, J. and Ketterman, A. J. (1996) Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (species B). Insect Biochem. Molec. Biol. **26**, 277–285
18 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
19 Dragani, B., Stenberg, G., Melino, S., Petruzzelli, R., Mannervik, B. and Aceto, A. (1997) The conserved N-capping box in the hydrophobic core of glutathione S-transferase P1-1 is essential for refolding. Identification of a buried and conserved hydrogen bond important for protein stability. J. Biol. Chem. **272**, 25518–25523
20 Cocco, R., Stenberg, G., Dragani, B., Principe, D. R., Paludi, D., Mannervik, B. and Aceto, A. (2001) The folding and stability of human alpha class glutathione transferase A1-1 depend on distinct roles of a conserved N-capping box and hydrophobic staple motif. J. Biol. Chem. **276**, 32177–32183
21 Kong, G. K. W., Polekhina, G., McKinstry, W. J., Parker, M. W., Dragani, B., Aceto, A., Paludi, D., Principe, D. R., Mannervik, B. and Stenberg, G. (2003) Contribution of glycine 146 to a conserved folding module affecting stability and refolding of human glutathione transferase P1-1. J. Biol. Chem. **278**, 1291–1302
22 Rossjohn, J., McKinstry, W. J., Oakley, A. J., Parker, M. W., Stenberg, G., Mannervik, B., Dragani, B., Cocco, R. and Aceto, A. (2000) Structures of thermolabile mutants of human glutathione transferase P1-1. J. Mol. Biol. **302**, 295–302
23 Stenberg, G., Dragani, B., Cocco, R., Mannervik, B. and Aceto, A. (2000) A conserved "hydrophobic staple motif" plays a crucial role in the refolding of human glutathione transferase P1-1. J. Biol. Chem. **275**, 10421–10428
24 Sayed, Y., Wallace, L. A. and Dirr, H. W. (2000) The hydrophobic lock-and-key intersubunit motif of glutathione transferase A1-1: implications for catalysis, ligandin function and stability. FEBS Lett. **465**, 169–172
25 Stenberg, G., Abdalla, A.-M. and Mannervik, B. (2000) Tyrosine 50 at the subunit interface of dimeric human glutathione transferase P1-1 is a structural key residue for modulating protein stability and catalytic function. Biochem. Biophys. Res. Comm. **271**, 59–63

26 Segel, I. H. (1993) Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady state enzyme systems. John Wiley & Sons, New York
27 Stenberg, G., Board, P. G., Carlberg, I. and Mannervik, B. (1991) Effects of directed mutagenesis on conserved arginine residues in a human class alpha glutathione transferase. Biochem. J. **274**, 549–555
28 Labrou, N. E., Mello, L. V. and Clonis, Y. D. (2001) Functional and structural roles of the glutathione-binding residues in maize (*Zea mays*) glutathione S-transferase I. Biochem. J. **358**, 101–110
29 Nieslanik, B. S. and Atkins, W. M. (2000) The catalytic Tyr-9 of glutathione S-transferase A1-1 controls the dynamics of the C terminus. J. Biol. Chem. **275**, 17447–17451
30 Jemth, P. and Mannervik, B. (2000) Active site serine promotes stabilization of the reactive glutathione thiolate in rat glutathione transferase T2-2. Evidence against proposed sulfatase activity of the corresponding human enzyme. J. Biol. Chem. **275**, 8618–8624
31 Manoharan, T. H., Gulick, A. M., Puchalski, R. B., Servias, A. L. and Fahl, W. E. (1992) Structural studies on human glutathione S-transferase. Substitution mutations to determine amino acids necessary for binding glutathione. J. Biol. Chem. **267**, 18940–18945
32 Tan, K.-L., Chelvanayagam, G., Parker, M. W. and Board, P. G. (1996) Mutagenesis of the active site of the human theta-class glutathione transferase GSTT2: catalysis with different substrates involves different residues. Biochem. J. **319**, 315–321

Received 12 March 2003/11 April 2003, accepted 23 April 2003
Published as BJ Immediate Publication 23 April 2003, DOI 10.1042/BJ20030394

ELSEVIER

Insect Biochemistry and Molecular Biology 33 (2003) 971–979

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Non-active site residues Cys69 and Asp150 affected the enzymatic properties of glutathione *S*-transferase AdGSTD3-3

Jeerang Wongtrakul, Rungrutai Udomsinprasert, Albert J. Ketterman *

Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand

Received 13 January 2003; received in revised form 5 June 2003; accepted 5 June 2003

Abstract

To elucidate how non-active site residues support the catalytic function, five selected residues of AdGSTD3-3 isoenzyme were changed to AdGSTD1-1 residues by means of site-directed mutagenesis. Analysis of the kinetic parameters indicated that Cys69Gln and Asp150Ser showed marked differences in V_{max} and K_m compared with the wild type enzyme. Both residues were characterized further by replacement with several amino acids. Both the Cys69 and Asp150 mutants showed differences with several GST substrates and inhibitors including affecting the interactions with pyrethroid insecticides. Cys69 and Asp150 mutants possessed a decreased half-life relative to the wild type enzyme. The Asp150 mutation appears to affect neighboring residues that support two important structural motifs, the N-capping box and the hydrophobic staple motif. The Cys69 mutants appeared to have subtle conformational changes near the active site residues resulting in different conformations and also directly affecting the active site region. The results show the importance of the cumulative effects of residues remote from the active site and demonstrate that minute changes in tertiary structure play a role in modulating enzyme activity.

Keywords: Site-directed mutagenesis; Enzyme characterization; Protein stability; Molecular modeling

1. Introduction

Glutathione *S*-transferases (GSTs) (E.C 2.5.1.18) are multifunctional enzymes involved in the detoxification and excretion of physiological and xenobiotic substances. The enzymes function by catalyzing the nucleophilic addition of the thiol of reduced glutathione to electrophilic centers in organic compounds (Booth et al., 1961). These proteins have the ability to bind large molecules for storage and transport roles using parts of the substrate binding sites (Oakley et al., 1999). GSTs also have been shown to regulate the stress kinase by acting as Jun-N terminal kinase (JNK) inhibitors (Adler et al., 1999). An additional signaling role shown for these cytosolic enzymes is modulation of ryanodine receptors, which are calcium channels (Dulhunty et al., 2001). Polymorphic GSTs are found in many organisms and organized into many classes e.g. alpha, mu, pi, theta, sigma, delta and kappa based on substrate and inhibitor specificity, immunological property and amino acid sequences (Mannervik and Danielson, 1988; Mannervik et al., 1992; Mannervik, 1985; Armstrong, 1997; Ketterer, 2001).

All of the cytosolic GSTs have the same basic protein fold, which consists of two domains. The N-terminal domain consists of beta sheets and alpha helices. The major function of this domain is to provide the G site, the binding site for glutathione (GSH). The C-terminal domain is an all α-helical domain. It provides structural elements for the recognition of hydrophobic substrates; the H site, and helps to define the substrate selectivity of the enzymes (Board et al., 2000; Ji et al., 1995; Reinemer et al., 1992; Reinemer et al., 1996; Sinning et al., 1993; Wilce et al., 1995). Although dimeric GSTs have a canonical tertiary structure, the subunit interface of the class alpha, mu and pi is characterized by a ball and socket hydrophobic interaction. Whereas, the interface

* Corresponding author. Fax: +66-2-441-9906.

Abbreviations: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; AdGSTD3-3, *An. dirus* GST delta 3-3; AdGSTD1-1, *An. dirus* GST delta 1-1

E-mail address: albertketterman@yahoo.com (A.J. Ketterman).

0965-1748/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0965-1748(03)00103-6

for the sigma, theta and delta enzymes is flatter, more hydrophilic and lacks the lock-and-key motif (Board et al., 2000; Ji et al., 1995; Reinemer et al., 1992; Reinemer et al., 1996; Sinning et al., 1993; Wilce et al., 1995; Oakley et al., 2001).

At present, several of the G site and H site residues have been studied in mammalian GST classes (Dirr et al., 1994). However, non-active site residues also have effects on the properties of the enzymes through structural effects. Several studies have demonstrated that a single amino acid change outside of the active site could affect the catalytic activity of GSTs. In pi class, the conserved residues at the N-terminus of α-6, an N-capping box motif, has a role in folding and stability. Several mutants at this position showed an increase in k_{cat} and $k_{cat}/K_{m\ CDNB}$ of approximately 1.5-fold (Dragani et al., 1997). In alpha class, Phe 51 has a role in stabilizing the conformation of the dimeric protein through a lock-and-key intersubunit interaction. The mutation of Phe 51 to serine caused a change in binding affinity toward GSH and 1-chloro-2,4-dinitrobenzene (CDNB) substrates (Sayed et al., 2000). In addition, there is an intradomain interaction formed by the side chain of Trp 20 into a hydrophobic pocket in domain 2. The mutation of Trp 20 to alanine reduced the specific activity of GSTA 1-1 (Wallace et al., 2000). Another example of structural residue effects is the mutation of Cys 101 to serine in a pi class GST that increased the binding affinity toward CDNB by inducing a conformational change in α-4 which contains several H-site residues (Park et al., 2001). A study with a delta class GST shows that residue Glu 25 of AdGSTD1-1 effects the hydrophobic core in domain 1, whereas Ala 188 is a part of the hydrophobic core in domain 2 and mutations at these two positions changed the catalytic activity of the enzymes (Ketterman et al., 2001).

Insect GSTs are of particular interest because they may be involved in insecticide resistance. The *adGSTIAS1* gene identified from an *Anopheles dirus* genomic library, expresses alternatively spliced products that yield four mature transcripts (Pongjaroenkit et al., 2001). The four splicing products, adGST1-1, adGST1-2, adGST1-3 and adGST1-4, have been cloned and characterized (Jirajaroenrat et al., 2001; Ketterman et al., 2001). Previously the four splicing products were named according to the insect GST nomenclature in use. However, to be in alignment with a proposed universal GST nomenclature we have renamed the enzymes AdGSTD1-1, AdGSTD2-2, AdGSTD3-3 and AdGSTD4-4 (Chelvanayagam et al., 2001). Where the 'D' refers to the GST delta class and the subunit number remains the same while 3-3, for example, signifies the enzyme is a homodimer. It was found that the AdGSTD3-3 isoenzyme could catalyze CDNB conjugation with a rate of 60 μmol/min/mg whereas the AdGSTD1-1 isoenzyme had a rate of 12 μmol/min/mg. Both isoenzyme subunits are 209 amino acids and share a 77% amino acid identity. From the 26 non-conserved residues in these GSTs, five residues were initially chosen, Cys69, Asp150, His178, Thr186 and Tyr206. These five were selected because of the differences in amino acid properties and the positioning of the side chain of the residues which face into the protein as shown by the tertiary structure of AdGSTD3-3 isoenzyme (Oakley et al., 2001). All of the residues are located outside of the active site and in domain II except Cys69 which is in domain I (Oakley et al., 2001). Therefore this work aimed to study the structure–function relationship of residues outside the active site of an insect GST by identifying important residues in AdGSTD3-3. All mutants were expressed and then characterized using several kinetic parameters, thermal stability and molecular modeling.

2. Materials and methods

2.1. Oligonucleotide-directed mutagenesis

The DNA encoding AdGSTD3-3 in pET3a (Jirajaroenrat et al., 2001) was used as a template in the mutagenesis procedure. The single mutations Cys69Gln, Cys69Asn, Cys69Ala, Asp150Ser, Asp150Tyr, Asp150Ala, His178Asn, Thr186Cys, Tyr206Lys and the double mutations Cys69Gln/Asp150Ser were made with the following oligonucleotides: Cys69Gln: 5′GCG CGC CAT CCA GAC GTA CTT AGC GGA GAA GTA CGG CAA G 3′, Cys69Asn: 5′CTG TGG GAG TCG CGC GCC ATC AAT ACG TAC TTG GCG GAG 3′, Cys69Ala: 5′GAG TCG CGC GCC ATC GCT ACG TAC TTG GCG GAG AAG 3′, Asp150Ser: 5′GCA CAA GTA CGT GGC GGG CTC GAG TCT GAC GAT CGC G 3′, Asp150Tyr: 5′GGG CAC AAG TAC GTA GCG GGC GCG AGT ACG ATC GCG 3′, Asp150Ala: 5′GGG CAC AAG TAC GTA GCG GGC GCG AGT ACG ATC 3′, His178Asn: 5′ GGG CTT CGA GCT CGC GAA GTA CCC GAA TGT GGC GGC GTG GTA 3′, Thr186Cys: 5′ TACGAG CGC TGT CGC AAG GAG GCG CCC GGT GCC GCC AT 3′, Tyr206Lys: 5′ GAG GAG TTC AGG AAG AAA TTC GAA AAG TAA CAT ATG GCT AGC 3′. The QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) was used in accordance with the manufacturer's instructions. Clones with the required mutation were first identified by restriction digest of the plasmids, and confirmed by full-length sequencing in both directions using a BigDye™ Terminator Cycle Sequencing Kit (Perkin–Elmer).

2.2. Expression and purification of wild type and mutant AdGSTD3-3 isoenzymes

A colony of *E. coli* BL21 (DE3)pLysS which contained a recombinant plasmid was grown at 37 °C until

the OD_{600} was approximately 0.6. IPTG was added to a final concentration of 0.1 mM, then the incubation was prolonged for a further 3 h. The purification of enzymes was performed as follows. The bacterial cells from 200 ml culture were collected by centrifugation at 5000 rpm, 4 °C for 10 min. The pellets were resuspended in 19.2 ml of PBS, pH 7.3 (140 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 of 50 mM Tris–HCl pH 7.4, 1 mM EDTA), 800 μl of 100 mg/ml lysozyme and 14.4 μl of 1.4 M β-mercaptoethanol were then incubated on ice for 30 min. 200 μl of 1 M DTT was added and the mixture was lysed at 900 psi in a French Pressure cell. The particulate material was removed by centrifugation at 10,000*g* at 4 °C for 20 min. The resulting supernatant was subjected to GSTrap affinity column. All the steps to obtain the purified GSTs were performed at 4 °C. The non-specific binding proteins were removed by washing with PBS buffer. The proteins bound to the column were eluted with 50 mM Tris–HCl (pH 8.0), containing 10 mM reduced–glutathione and 10 mM DTT. The fractions containing recombinant GST were pooled before concentrating by using a centriprep-10 (Amicon) at 2500*g*, 4 °C. Then the bound glutathione was eliminated using HiTrap desalting column (Amersham Pharmacia Biotech) equilibrated with 50 mM phosphate buffer pH 6.5. The concentrated protein was applied to the column and eluted in the same buffer containing 10 mM DTT. The concentration step was repeated until the final volume was 0.5 ml. Glycerol was added to a final concentration of 50% and the purified concentrated GSTs were stored at −20 °C.

2.3. Characterization of the expressed enzymes

The purity of an enzyme preparation was determined by SDS-PAGE using Bio-Rad low range standards as molecular weight markers. K_m and V_{max} for 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) were determined by non-linear regression analysis using Graphpad Prism 2.01 Software. Specific activities toward several GST substrates were determined spectrophotometrically. The GST activities were measured with glutathione and five other substrates; 1-chloro-2,4-dinitrobenzene (CDNB), 1-2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid, 4-nitrophenethyl bromide and 4-nitrobenzyl chloride using the appropriate pH and λ_{max} (Habig et al., 1974). To determine the inhibition kinetics parameter K_i for the GSTs the effect of cumene hydroperoxide, *S*-hexylglutathione and the pyrethroid insecticides, deltamethrin and permethrin, on the CDNB conjugating activity of GSTs was determined in assays at fixed concentration of 10 mM GSH containing varying concentrations of CDNB 0.02–2.5 mM in 0.1 M phosphate pH 6.5. The K_i results were calculated based on double-reciprocal Lineweaver–Burk plots. In the thermal stability assay, the enzyme was incubated 10 min at different temperatures ranging from 30 to 50 °C at a protein concentration of 0.1 mg/ml in 0.1 M potassium phosphate (pH 6.5) containing 5 mM DTT and 1 mM EDTA. The enzyme was heated in sealed eppendorf tubes and the temperature was monitored with a thermometer. The stability time courses were determined by withdrawing suitable aliquots at different time points from the denaturation mixture for assay of remaining activity. Protein concentration was determined using the Bio-Rad protein reagent with bovine serum albumin as the standard protein (Bradford, 1976).

2.4. Molecular modeling

Initial coordinates of AdGSTD3-3 were taken from the X-ray crystal structure of the complex with glutathione (Oakley et al., 2001). Modeling of the tertiary structure was performed using the Insight II software (Biosym/MSI, San Diego, CA). Initial strains in the system were released by a two-step energy minimization, using the steepest descent and conjugate gradient algorithm until the average absolute derivative was 10^{-7}. Then the models of the mutants were generated. The minimization was repeated to obtain the most stable model. To study the effects on tertiary structure of the protein, several parameters were measured e.g. root mean square deviation of the alpha carbon backbone (rms) using swisspdb software, distances across the active site pocket, dihedral angle changes, residue movements, H-bond distances and H-bond angles.

3. Results

3.1. Expression and purification of mutant enzymes

To assess the contribution of the five chosen residues to the differences between AdGSTD3-3 and AdGSTD1-1, five mutants of AdGSTD3-3; Cys69Gln, Asp150Ser, His178Asn, Thr186Cys and Tyr206Lys were generated by PCR with a single amino acid being replaced with the equivalent amino acid in AdGSTD1-1. The locations of the five residues are shown in Fig. 1. Mutants and wild type enzymes were then expressed in *E. coli* and purified by affinity chromatography on immobilized GSH. The purified proteins gave a single band on SDS-polyacrylamide gel electrophoresis with a size of approximately 23 kDa, which corresponds to the calculated molecular weight of the GST subunits.

3.2. Kinetic properties

Comparison of the kinetic parameters of AdGSTD3-3 mutants with the wild type values showed that the residue changes affected enzymatic properties (Table 1). Three of the mutant GSTs, His178Asn, Thr186Cys and

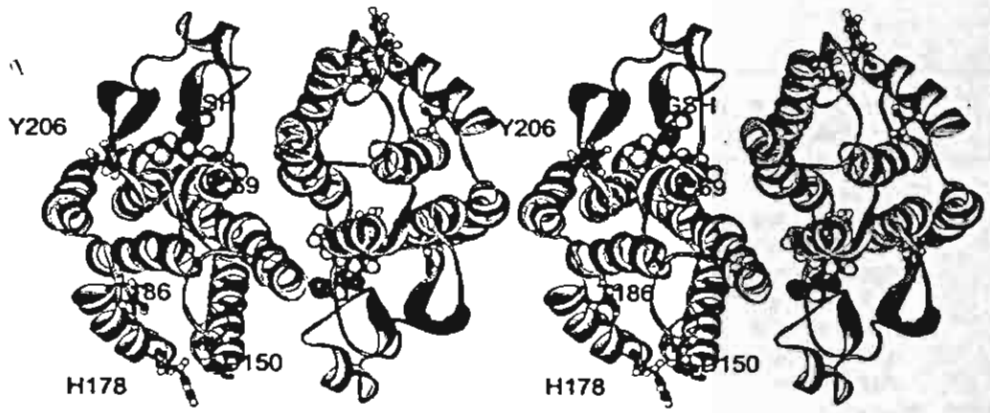


Fig. 1. Stereoview of homodimeric AdGSTD3-3 complexed with glutathione. The five selected residues are labeled and shown in ball-and-stick form.

Table 1
Kinetic constants for the AdGSTD3-3 mutants

Enzyme	V_{max}	k_{cat}	GSH		CDNB	
			K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
Wild type AdGSTD3-3	63.6 ± 6.2	25.2	0.29 ± 0.03	86.9	0.13 ± 0.01	194
Wild type AdGSTD1-1*	12.9 ± 0.6	5.03	0.86 ± 0.18	5.86	0.10 ± 0.03	48.4
Cys69Gln	105 ± 7	41.7	0.44 ± 0.03	96.9	0.21 ± 0.03	198
Asp150Ser	70.2 ± 1.0	27.8	0.17 ± 0.02	160	0.07 ± 0.01	391
His178Asn	67.4 ± 2.8	26.7	0.51 ± 0.05	53.6	0.12 ± 0.01	222
Thr186Cys	59.5 ± 3.4	23.6	0.31 ± 0.04	76.8	0.12 ± 0.02	195
Tyr206Lys	63.5 ± 3.3	25.1	0.22 ± 0.02	114	0.25 ± 0.05	100
Cys69Asn	26.7 ± 6.4	9.61	0.53 ± 0.03	18.2	0.08 ± 0.01	117
Cys69Ala	65.5 ± 7.5	25.9	0.33 ± 0.04	79.2	0.11 ± 0.03	235
Asp150Tyr	74.2 ± 6.8	29.5	0.21 ± 0.02	141	0.09 ± 0.01	327
Asp150Ala	80.2 ± 7.0	31.7	0.33 ± 0.02	96.7	0.08 ± 0.02	382
Cys69Gln/Asp150Ser	116 ± 7	46.1	0.40 ± 0.04	117	0.23 ± 0.03	200
Arg96Ala	118 ± 7	46.8	2.75 ± 0.12	17	0.38 ± 0.06	125

The units are: V_{max}, μmole/min/mg; K_m, mM; k_{cat}, s^{-1}, k_{cat}/K_m, mM^{-1} s^{-1}. The data are mean ± standard deviation from at least three independent experiments.

* See Ketterman et al. (2001)).

Tyr206Lys, had the same maximal velocity (V_{max}) as the wild type but the replacement of Cys69 with glutamine caused a significant increase of 65% in the V_{max}. There were approximately 1.7- and 1.8-fold decreases in the K_m value for GSH and CDNB for the mutant Asp150Ser. The k_{cat}/K_m for Asp150Ser shows that it possesses about a 1.8- and 2-fold greater catalytic efficiency for both GSH and CDNB, respectively, compared to the wild type enzyme. In contrast, Tyr206Lys had the lowest efficiency for CDNB and His178Asn had the lowest toward GSH. Only the two positions Cys69 and Asp150 were characterized further. Amino acid substitutions were performed producing Cys69Asn, Cys69Ala, Asp150Tyr and Asp150Ala. In addition, the double mutant Cys69Gln/Asp150Ser, was also generated to examine the possible combined effect of both residues (Table 1). It was found that the alteration of Cys69 with asparagine significantly decreased the k_{cat} 2.6-fold compared to the wild type AdGSTD3-3. However, there was no significant change observed in the kinetic parameters of Cys69Ala. The double mutant enzyme Cys69Gln/Asp150Ser possessed kinetic constants very similar to those observed for the single mutant Cys69Gln. Kinetic constants for the single mutant Cys69Gln and Cys69Asn strongly suggest that the amino acid at position 69 has a major structural influence on the catalytic activity of the enzymes. The Asp150Tyr mutant showed a slightly lower $K_{m\ GSH}$ than the wild type AdGSTD3-3 whereas Asp150Ala had a greater value. However both mutants displayed a significantly lower K_m for CDNB, as did the Asp150Ser mutant.

To establish whether the mutant and wild type enzymes have similar or distinct catalytic specificity, their activities toward a panel of established GST substrates; (1-chloro-2,4-dinitrobenzene (CDNB), 1-2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA),

Table 2
Specific activity of the AdGSTD3-3 mutants towards five different substrates

Enzyme	CDNB (1 mM)	DCNB (1 mM)	4-Nitrobenzyl chloride (0.1 mM)	4-Nitrophenethyl bromide (0.1 mM)	Ethacrynic acid (0.2 mM)
Wild type AdGSTD3-3	57.2 ± 3.2	0.248 ± 0.003	0.135 ± 0.013	0.019 ± 0.007	0.087 ± 0.010
Cys69Gln	95.6 ± 2.0	0.249 ± 005	0.165 ± 0.013	0.070 ± 0.008	0.126 ± 0.016
Cys69Ala	50.2 ± 0.9	0.205 ± 0.003	0.196 ± 0.025	0.057 ± 0.003	0.109 ± 0.022
Cys69Asn	32.0 ± 0.6	0.252 ± 0.007	0.165 ± 0.024	0.030 ± 0.015	0.064 ± 0.006
Asp150Ser	64.7 ± 5.5	0.249 ± 0.005	0.142 ± 0.009	0.070 ± 0.008	0.126 ± 0.016
Asp150Ala	68.9 ± 3.8	0.284 ± 0.009	0.111 ± 0.004	0.021 ± 0.004	0.050 ± 0.017
Asp150Tyr	63.9 ± 3.7	0.289 ± 0.003	0.173 ± 0.020	0.067 ± 0.013	0.100 ± 0.016
Cys69Gln/Asp150Ser	97.7 ± 7.7	0.315 ± 0.002	0.209 ± 0.006	0.069 ± 0.017	0.100 ± 0.009

The units are μmole/min/mg of protein. The data are mean ± standard deviation from at least three independent experiments.

4-nitrophenethyl bromide and 4-nitrobenzyl chloride) were determined (Table 2). The Cys69 mutants showed differences with several of the GST substrates especially CDNB and EA. The Asp150 mutants displayed several differences in the conjugating activity towards the tested substrates most notably with 4-nitrophenethyl bromide and EA. The double mutant enzyme had a significant increase in the DCNB, 4-nitrobenzyl chloride and 4-nitrophenethyl bromide activity compared to the wild type AdGSTD3-3. It also possessed greater activity towards DCNB and 4-nitrobenzyl chloride than the respective single residue mutant enzymes. An inhibition kinetics parameter, K_i, for adGSTD3-3 mutants was determined using four different compounds, cumene hydroperoxide, deltamethrin, permethrin and *S*-hexylglutathione (Table 3). The K_i ranges of the GSTs varied from 3-fold for deltamethrin up to 80-fold for *S*-hexylglutathione. All mutations increased the affinity of interaction, relative to the wild type enzyme, up to 30-fold with CuOOH but decreased the affinity up to 5-fold for interaction with permethrin. Of special interest was the Asp150Ala mutant which displayed the greatest affinity, lowest K_i value, for deltamethrin but the lowest affinity, greatest K_i value, for permethrin demonstrating conformational differences in the adGSTD3-3 are involved in governing the binding for the pyrethroid insecticides.

The stability experiment was performed at different temperatures and demonstrated that the wild type and most of the mutant enzymes were stable up to 40 °C. The half-life of the enzymes were calculated based on the relationship between the log percentage of original activity incubated at 4 °C and the activity at different temperatures of preincubation. The half-life of the wild type and Cys69Gln at 40 °C was approximately 31.9 and 40 min whereas both Cys69Ala and Cys69Asn possessed a shorter half-life, of 28.8 and 1 min(s), respectively. At 45 °C, Cys69Asn lost all activity, however the wild type, Cys69Gln and Cys69Ala retained their activities each with a half-life of approximately 6.2, 1.8 and 1.5 min, respectively. The results suggest that the amino acid at position 69 affects the core residues in domain 1, the mutational changes thereby disrupting the enzyme stability. The Asp150 mutants and the Cys69Gln/Asp150Ser double mutant were relatively stable to incubation at temperatures up to 40 °C. The exception was the Asp150Tyr mutant, which lost all activity at 40 °C. At 45 °C the mutants Cys69Gln/Asp150Ser, Asp150Ser and Asp150Ala showed a decrease in half-life to 1.9, 1.6 and

Table 3
Determination of K_i for AdGSTD3 mutants

Enzymes	K_i (μM)			
	CuOOH	Deltamethrin	Permethrin	*S*-Hexyl
adGSTD3-3	34,900	48.1	31.3	3.1
Cys69Gln	1300	63.7	75.2	3.8
Cys69Ala	23,300	50.1	47.0	2.9
Cys69Asn	1200	61.8	51.0	3.0
Asp150Ser	13,100	56.0	39.0	97
Asp150Ala	8500	21.2	166	4.1
Asp150Tyr	8300	40.1	46.1	4.4
Cys69Gln/Asp150Ser	23,000	59.7	128	231

CuOOH is cumene hydroperoxide, and *S*-hexyl is *S*-hexylglutathione.

0.94 min, respectively. Therefore the amino acid at position 150 also appeared to influence the enzyme structure as demonstrated by the stability effects.

3.3. Molecular modeling

After the residues of the wild type were replaced and the energy of the system was minimized to an average absolute derivative of 10^{-7} then the reliability of the model was tested by changing a mutant residue back to the wild type. The energy of the system was re-minimized to an average absolute derivative of 10^{-7}. Two parameters; dihedral angle and residue movement of the two minimized wild type structures, were compared and only very small changes were observed; 0.93° and 0.3 Å, respectively indicating a relatively good reproducibility of the model.

The analysis of the three dimensional models of the Cys69Gln, Cys69Ala and Cys69Asn indicated that the root mean square deviation of the alpha carbon backbone (rms) ranged from 0.01 to 0.74 Å suggesting that the alteration of Cys69 caused no effects on the main chain of the protein. Another parameter measured was the dihedral angle. All the Cys69 mutants showed dihedral angle changes of approximately 0.02–2.03° in helix 1 to helix 8 suggesting that there was no significant change in conformation. The distances across the active site were measured by selecting 10 pairs of residues that line the active site. The changes in distance were less than 1 Å therefore the alteration of the residues did not appear to change the conformation of the active site. All the H-bond distances and H-bond angles were measured within one GST subunit. The range of the H-bond distance was 1.6–2.6 Å whereas the range of the H-bond angle was 110–180°. All of the Cys69 models showed no differences in H-bond distances but they demonstrated greater changes in the main chain H-bonds which were 18.27°, 8.47°, 7.12° in Cys69Gln, Cys69Ala and Cys69Asn, respectively. The residue movements were measured from the alpha carbon of the residue 69 to all the neighboring residues within 25 Å range of the dimeric GST model. All the Cys69 models showed small movements in the atoms ranging from 0.3 to 1.67 Å.

In the Asp150 mutant models including the double mutant Cys69Gln/Asp150Ser, the rms of the α carbon backbone, the active site distances and the H-bond distances were in the same ranges as the Cys69 mutants. However, the remaining parameters showed greater changes compared to the wild type model. The dihedral angle of helix 7 in the Asp150Tyr, Asp150Ser and Cys69Gln/Asp150Ser models was changed approximately 5°. In addition, the dihedral angle of Asp150Ala was changed 34° in helix 7 and 7–8° in helix 5 and 6. All the results strongly suggest that there are conformational changes in the Asp150 mutants in domain 2. The residue movements of Asp150Tyr and Asp150Ser mutants revealed maximum changes in the distances measured to His144, located in the loop before helix 6, of approximately 2.25 and 1.48 Å. Whereas the Asp150Ala mutant showed a maximum change of 2.65 Å measured to Gln189 which is located in helix 7. These results provide further evidence of subtle conformational changes of the Asp150 mutants. The maximum movement of Cys69Gln/Asp150Ser was 1.78 Å when measured from the α carbon of residue 150 to Thr18 and 1.29 Å when measured from the α carbon of residue 69 in one subunit to residue 69 in the other subunit. The maximum changes in H-bond angles were also found in domain 2 of Asp150 mutants. In each model the maximum changes ranged from 4.5 to 18.7° and the involved residues, Leu141, Gly143, Lys145, Arg185 and Glu189, were located in similar positions in helix 7 and the loop before helix 6. However, the Cys69Gln/Asp150 mutant demonstrated a maximum change in the main chain H-bond angle in domain 1 that forms between the NH of Thr18 and the oxygen from Ala14 of approximately 18.54° which was similar to Cys69Gln.

4. Discussion

Characterization of the mutant enzymes of the first five residues initially chosen demonstrated that single residue changes affected the enzymatic property. The mutations at position 69 and 150 appeared to affect sensitive regions of the tertiary structure resulting in changes in V_{max} and substrate specificity of the enzyme as shown in Tables 1 and 2. Additional five mutations of the two residues showed differences in substrate specificity, percent inhibition and thermal stability when compared to the wild type AdGSTD3-3.

To aid in interpretation of the observed kinetic data, six modeling parameters were explored using molecular modeling. It was found that three parameters; root mean square deviation of the alpha carbon backbone, the configuration of the active site, and the hydrogen bond distance did not change significantly. However, subtle conformational changes can be observed from the remaining three parameters; the dihedral angles of the helices, residue movements and hydrogen bond angles suggesting that the change in the kinetic data occurs due to differing conformational changes of the enzymes.

The modeling data suggests that the changes in the Cys69 mutant kinetics occur from subtle conformational shifts. Among the six parameters observed, only H-bond angles showed the greatest changes. Based on the models of Cys69Asp, Cys69Ala and Cys69Gln, the NH group from the main chain of Cys69 formed an H-bond with the main chain carboxyl group of Ser65, which is an active site residue directly interacting with GSH. When compared to the wild type, those H-bond angles were changed approximately 5–8°. A second change is

between residue Thr18 and Ala14. The NH group from Thr18 forms an H-bond with the carboxyl group of Ala14. In the Cys69Gln and Cys69Gln/Asp150Ser models, these angles are changed approximately 18°. Both locations are near the active site residues 64–66 that directly interact with GSH. These changes suggest that atom movements occur that yield different conformations and directly affect the active site region. In addition, the residue changes also affect the packing of the hydrophobic core in domain 1 which is formed by the residues in helix 1 and helix 3 (Dirr et al., 1994). Cys69 is located in helix 3 and functions in structural stabilization. All the Cys69 mutants possessed a much shorter half-life at 45 °C. The mutation at residue 69 would affect the neighboring residues that interact with Ile68 such as Ala67 and Tyr71. Ile68 appears equivalent to an invariant isoleucine found in alpha, mu and pi class GST. Mutation of this isoleucine was shown to disrupt the hydrophobic core and inactivate the enzyme (Manoharan et al., 1992).

The effects of the Asp150 residue on the active site can be proposed to occur through two pathways based on the results from the models. The first pathway is an intra-subunit route. Asp150 forms an ionic interaction with His144, which showed maximum movement in the models. This interaction may stabilize the loop of residues 142–155 before helix 6. There are two conserved hydrophobic residues within the loop, Val147 and Ala148 (which are Ala147 and Ala148 in AdGSTD1-1; Val151 and Ala152 in AdGSTD2-2; Val155 and Ala156 in AdGSTD4-4; Val147 and Ala148 in *L. cuprina*; Ala148 and Ala149 in *D. melanogaster*). These two hydrophobic residues have atom–atom interactions with neighboring residue Arg96 in helix 4. The varying interactions in the different mutants would generate positional differences in Arg96 that would then affect interactions with Arg66, which is an active site residue. The other possible pathway is between subunits of the protein. The changes in residue interactions affecting Arg96 would also effect the interaction with Trp63 in the other GST subunit. The positional changes in Trp63 would then influence the interaction with Gln49, which is an active site residue. There is evidence of signaling across the GST subunits through an interaction of Arg69 and Gln100 with the active site residue Arg15 in alpha class (Xiao et al., 1999).

To test the hypotheses the mutant Arg96Ala, a pivotal residue shared by both proposed pathways, was generated. It showed dramatic effects on the enzyme affinities and stability. The change in k_{cat} for Arg96Ala was only increased approximately 2-fold compared to the wild type AdGSTD3-3 (Table 1). However, the K_m for CDNB increased 3-fold and the K_m for GSH increased approximately 10-fold. In addition, Arg96Ala possessed a half-life 30-fold greater, 187 min compared to 6.2 min for the wild type. Therefore Arg96Ala appeared to be involved with both catalysis and structural support. However, the major residues involved in affecting Arg96 via the two proposed pathways remain to be elucidated.

The AdGSTD Asp150 mutation also would affect neighboring residues that generate two important motifs, the N-capping box and the hydrophobic staple motif. These motifs are located in the loop before helix 6 and play a major role in the folding and stability of GSTs. The N-capping box motif consists of Thr153 and Asp156 that H-bond to each other. In addition, Val147 also forms an H-bond with Asp156. Mutations at these positions in pi class destabilized the enzyme structure at higher temperatures by disrupting all the hydrogen bonding network (Dragani et al., 1997; Rossjohn et al., 2000). Based on the structure of the wild type AdGSTD3-3, Tyr146, Ala148 and Gly149 are analogous residues with similar H-bond interactions that generate this motif. Several atom movements observed from the Asp150 mutant models might lead to the loss of these interactions that would result in more disordering of the loop and destabilization of helix 6. The hydrophobic staple motif in AdGSTD3-3 consists of Leu152 and Leu157 located adjacent to the N-capping box motif. Several residues in the loop e.g. Thr153, Ile154 and Ala155 contact Leu152 and Leu157 both of which directly interact with many residues which are important structural elements influencing the active site residues in helix 1. A single mutation in either of Leu157 or Leu152 at the equivalent residues in alpha and pi class increased the k_{cat} and k_{cat}/K_m values for CDNB as well as destabilizing the enzymes (Cocco et al., 2001; Stenberg et al., 2000). Our Asp150 mutants e.g. Asp150Ser also showed a similar result suggesting that the residue interactions induce conformational changes in this region that destabilize helix 6 and affect the active site residues in helix 1 that result in changes in both the stability property of the protein and kinetic parameters. Further support for tertiary structure changes is given by the modeling data. The Asp150 mutants showed a large change in dihedral angles of helices 5–7 that would affect the packing of the hydrophobic core in domain 2. When the loop becomes less flexible, the residues on the loop would be packed in the hydrophobic core with less movement thereby yielding more stability and giving the wild type enzyme a longer half-life at 45 °C.

In our study, when the residues from isoenzyme AdGSTD3-3 were replaced with residues from isoenzyme AdGSTD1-1, the GST mutants Cys69Gln and Asp150Ser did not show any kinetic properties of isoenzyme AdGSTD1-1 as expected (Table 1), demonstrating that contributions from other residues are also needed. A mutation study in alpha class GST was performed to increase the activity of rat GST A1-1 toward 4-hydroxyalkenal similar to isoenzyme rGSTA4-4 (Björnestedt et al., 1995). It was shown that a substitution of Ala12Gly of GST A1-1 cannot increase the alkenal activity. A

chimeric rGSTA4-4 was generated by replacing the first 25 residues in the N-terminus with A1-1 residues resulting in a mutant enzyme with low alkenal activity. Then it was shown that the Ala12Gly point mutation of the chimeric enyzyme can confer high alkenal activity demonstrating that the non-active site residues are essential. In addition, human GST A2-2 was also improved in steroid isomerase activity by replacing five amino acids in the H-site with the residues of GST A3-3, the most efficient catalyst of double-bond isomerization (Pettersson et al., 2002; Johansson and Mannervik, 2001; Pettersson et al., 2002). These mutations resulted in a 3500-fold increase in catalytic efficiency with Δ^5-androstene-3, 17-dione which was less than the parental A3-3 by approximately 1500-fold, suggesting that the lower activity is from an indirect role of non-active site residues. An additional study succeeded in redesigning the substrate-selectivity of GST A1-1 to yield a high catalytic efficiency with toxic alkenal products of lipid peroxidation (Nilsson et al., 2000). The improved enzyme was generated by replacing four important active site residues and the C-terminus part containing several non-active site residues with the residues from GST A4-4. The mutant had a 300-fold increase in catalytic efficiency with nonenal compared to the original A1-1 and was 3.1-fold more efficient than the GST A4-4 that normally possessed the highest alkenal activity. However, the mutant still showed approximately 3-fold lower catalytic efficiency toward HNE when compared to GST A4-4 suggesting a role for other non-active site residues in influencing the catalytic residues in their required orientations.

Both amino acids Cys69 and Asp150 are non-active site residues, but their replacement appears to yield a large number of small changes in the enzyme that leads to different flexing or conformational changes thereby changing the enzymatic properties. This finding was also found in enzymes other than GSTs. For example, aspartate aminotransferase was mutated to a valine aminotransferase (Oue et al., 1999). This enzyme with 17 mutations demonstrated a 2.1×10^6-fold increase in k_{cat}/K_m for valine, a non-native substrate. However, only one of the mutated residues directly interacted with the substrate. The crystal structure demonstrated that the mutations affected the enzyme structure by changing the subunit interface including shifting of the enzyme domain that enclosed the substrate.

In conclusion, the influence of the non-active site residues in affecting the positioning of the catalytic residues depends on the specific amino acids involved including the surrounding milieu of amino acids. Therefore the results suggest the importance of the cumulative effects of residues remote from the active site and demonstrate that minute changes in tertiary structure play a role in modulating enzyme activity.

Acknowledgements

This work was supported by the Thailand Research Fund and Royal Golden Jubilee scholarships to J.W. and R.U.

References

Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J., Ronai, Z., 1999. Regulation of JNK signaling by GSTp. EMBO J. 18, 1321–1334.

Armstrong, R.N., 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem. Res. Toxicol. 10, 2–18.

Björnestedt, R., Tardioli, S., Mannervik, B., 1995. The high activity of rat glutathione transferase 8-8 with alkene substrates is dependent on a glycine residue in the active site. J. Biol. Chem. 270, 29705–29709.

Board, P.G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L.S., Schulte, G.K., Danley, D.E., Hoth, L.R., Griffor, M.C., Kamath, A.V., Rosner, M.H., Chrunyk, B.A., Perregaux, D.E., Gabel, C.A., Geoghegan, K.F., Pandit, J., 2000. Identification, characterization, and crystal structure of the omega class glutathione transferases. J. Biol. Chem. 275, 24798–24806.

Booth, J., Boyland, E., Sims, P., 1961. An enzyme from rat liver catalysing conjugations with glutathione. Biochem. J. 79, 516–524.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chelvanayagam, G., Parker, M.W., Board, P.G., 2001. Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. Chem.-Biol. Interact. 133, 256–260.

Cocco, R., Stenberg, G., Dragani, B., Principe, D.R., Paludi, D., Mannervik, B., Aceto, A., 2001. The folding and stability of human alpha class glutathione transferase A1-1 depend on distinct roles of a conserved N-capping box and hydrophobic staple motif. J. Biol. Chem. 276, 32177–32183.

Dirr, H., Reinemer, P., Huber, R., 1994. X-ray crystal structures of cytosolic glutathione *S*-transferases, Implications for protein architecture, substrate recognition and catalytic function. Eur. J. Biochem. 220, 645–661.

Dragani, B., Stenberg, G., Melino, S., Petruzzelli, R., Mannervik, B., Aceto, A., 1997. The conserved N-capping box in the hydrophobic core of glutathione *S*-transferase P1-1 is essential for refolding, identification of a buried and conserved hydrogen bond important for protein stability. J. Biol. Chem. 272, 25518–25523.

Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G., Board, P., 2001. The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. J. Biol. Chem. 276, 3319–3323.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Ji, X., Von Rosenvinge, E.C., Johnson, W.W., Tomarev, S.I., Piatigorsky, J., Armstrong, R.N., Gilliland, G.L., 1995. Three-dimensional structure, catalytic properties, and evolution of a sigma class glutathione transferase from squid, a progenitor of the lens *S*-crystallins of cephalopods. Biochemistry 34, 5317–5328.

Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L., Ketterman, A.J., 2001. Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single Anopheles gene. Insect Biochem. Molec. Biol. 31, 867–875.

Johansson, A.-S., Mannervik, B., 2001. Human glutathione transferase A3-3, a highly efficient catalyst of double-bond isomerization in

the biosynthetic pathway of steroid hormones. J. Biol. Chem. 276, 32061–32065.

Ketterer, B., 2001. A bird's eye view of the glutathione transferase field. Chem.-Biol. Interact. 138, 27–42.

Ketterman, A.J., Prommeenate, P., Boonchauy, C., Chanama, U., Leetachewa, S., Promtet, N., Prapanthadara, L., 2001. Single amino acid changes outside the active site significantly affect activity of glutathione S-transferases. Insect Biochem. Molec. Biol. 31, 65–74.

Mannervik, B., 1985. The isoenzymes of glutathione transferase. Adv. Enzymol. Relat. Areas Mol. Biol. 57, 357–417.

Mannervik, B., Awasthi, Y.C., Board, P.G., Hayes, J.D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W.R., Pickett, C.B., Sato, K., Widersten, M., Wolf, C.R., 1992. Nomenclature for human glutathione transferases. Biochem. J. 282, 305–306.

Mannervik, B., Danielson, U.H., 1988. Glutathione transferases—structure and catalytic activity. CRC Crit. Rev. Biochem. 23, 283–337.

Manoharan, T.H., Gulick, A.M., Puchalski, R.B., Servias, A.L., Fahl, W.E., 1992. Structural studies on human glutathione S-transferase π. Substitution mutations to determine amino acids necessary for binding glutathione. J. Biol. Chem. 267, 18940–18945.

Nilsson, L.O., Gustafsson, A., Mannervik, B., 2000. Redesign of substrate-selectivity determining modules of glutathione transferase A1-1 installs high catalytic efficiency with toxic alkenal products of lipid peroxidation. Proc. Natl. Acad. Sci. USA 97, 9408–9412.

Oakley, A.J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A.J., Wilce, M.C.J., 2001. The crystal structures of glutathione S-transferases isozymes 1-3 and 1-4 from Anopheles dirus species B. Protein Sci. 10, 2176–2185.

Oakley, A.J., Lo Bello, M., Nuccetelli, M., Mazzetti, A.P., Parker, M.W., 1999. The ligandin (non-substrate) binding site of human pi class glutathione transferase is located in the electrophile binding site (H-site). J. Mol. Biol. 291, 913–926.

Oue, S., Okamoto, A., Yano, T., Kagamiyama, H., 1999. Redesigning the substrate specificity of an enzyme by cumulative effects of the mutations of non-active site residues. J. Biol. Chem. 274, 2344–2349.

Park, H.-J., Lee, K.-S., Cho, S.-H., Kong, K.-H., 2001. Functional studies of cysteine residues in human glutathione S-transferase P1-1 by site-directed mutagenesis. Bull. Korean Chem. Soc. 22, 77–83.

Pettersson, P.-L., Johansson, A.-S., Mannervik, B., 2002. Transmutation of human glutathione transferase A2-2 with peroxidase activity into an efficient steroid isomerase. J. Biol. Chem. 277, 30019–30022.

Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L., Ketterman, A.J., 2001. Genomic organization and putative promotors of highly conserved glutathione *S*-transferases originating by alternative splicing in Anopheles dirus. Insect Biochem. Molec. Biol. 31, 75–85.

Reinemer, P., Dirr, H.W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G., Parker, M.W., 1992. Three-dimensional structure of class π glutathione *S*-transferase from human placenta in complex with *S*-hexylglutathione at 2.8 Å resolution. J. Mol. Biol. 227, 214–226.

Reinemer, P., Prade, L., Hof, P., Neuefeind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Barrunik, H.D., Bieseler, B., 1996. Three-dimenstional structure of glutathione S-transferase from Arabidopsis thaliana at 2.2 Å resolution: structural characterization of herbicide-conjugating plant glutathione S-transferases and a novel active site architecture. J. Mol. Biol. 255, 289–309.

Rossjohn, J., McKinstry, W.J., Oakley, A.J., Parker, M.W., Stenberg, G., Mannervik, B., Dragani, B., Cocco, R., Aceto, A., 2000. Structures of thermolabile mutants of human glutathione transferase P1-1. J. Mol. Biol. 302, 295–302.

Sayed, Y., Wallace, L.A., Dirr, H.W., 2000. The hydrophobic lock-and-key intersubunit motif of glutathione transferase A1-1: implications for catalysis, ligandin function and stability. FEBS Lett. 465, 169–172.

Sinning, I., Kleywegt, G.J., Cowan, S.W., Reinemer, P., Dirr, H.W., Huber, R., Gilliland, G.L., Armstrong, R.N., Ji, X., Board, P.G., Olin, B., Mannervik, B., Jones, T.A., 1993. Structure determination and refinement of human Alpha class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. J. Mol. Biol. 232, 192–212.

Stenberg, G., Dragani, B., Cocco, R., Mannervik, B., Aceto, A., 2000. A conserved "hydrophobic staple motif" plays a crucial role in the refolding of human glutathione transferase P1-1. J. Biol. Chem. 275, 10421–10428.

Wallace, L.A., Burke, J., Dirr, H.W., 2000. Domain–domain interface packing at conserved Trp-20 in class α glutathione transferase impacts on protein stability. Biochem. Biophys. Acta 1478, 325–332.

Wilce, M.C.J., Board, P.G., Feil, S.C., Parker, M.W., 1995. Crystal structure of a theta-class glutathione transferase. EMBO J. 14, 2133–2143.

Xiao, B., Singh, S.P., Nanduri, B., Awasthi, Y.C., Zimniak, P., Ji, X., 1999. Crystal structure of a murine glutathione *S*-transferase in complex with a glutathione conjugate of 4-hydroxynon-2-enal in one subunit and glutathione in the other: evidence of signaling across the dimer interface. Biochemistry 38, 11887–11894.

Protein and Peptide Letters, Vol. 10, No. 5, pp. 441-448, 2003
Bentham Science Publishers Ltd.
0929-8665/03 $ 41.00 + .00

MULTIPLE ROLES OF GLUTATHIONE BINDING-SITE RESIDUES OF GLUTATHIONE S-TRANSFERASE

Ardcharaporn Vararattanavech and Albert J. Ketterman*

Institute Of Molecular Biology And Genetics, Mahidol University Salaya Campus, Salaya, Nakhon Pathom 73170, Thailand; *Email: frakt@mahidol.ac.th

Abstract: This study was designed to characterize residues in the glutathione binding site of AdGSTD4-4 from the mosquito malaria vector *Anopheles dirus*. The data revealed that Leu33, His38 and His50 each play a role in enzyme catalysis and glutathione binding. The mutants of these three residues also displayed differences in hydrophobic substrate specificity, suggesting that changes in the active site conformation occurred. Differences in conformations was also suggested by protein stability changes. These results indicate that residues in the glutathione binding site are not only important in the catalytic function but also play a role in the structural integrity of the enzyme.

Keywords: active site, *Anopheles dirus*, catalysis, glutathione transferase, mosquito, structure.

INTRODUCTION

Glutathione S-transferases (GSTs: E.C. 2.5.1.18) are intracellular proteins which are widely distributed in nature and found in most aerobic eukaryotes and prokaryotes. They are involved in xenobiotic metabolism as well as protection against peroxide damage [1, 2]. GSTs catalyze the S-conjugation between the thiol group of the tripeptide glutathione (GSH, γ-glutamyl-L-cysteinyl-glycine) and a large number of electrophilic moieties in the hydrophobic substrates [2-4]. A simplified enzyme catalysis scheme for GSTs first involves GSH substrate binding to the active site, then GSH ionization to form a nucleophilic thiolate anion (GS^-), followed by substrate conjugation, product formation and finally product release [5-7]. Once the GSH thiolate anion (GS^-) is formed in the binding site, it is capable of reacting spontaneously by nucleophilic attack with electrophilic compounds in close proximity. The conjugation increases the solubility of the target molecule thus facilitating the excretion of the molecule from the organism.

All cytosolic GSTs consist of two domains [5, 8-10]. The N-terminal domain constitutes roughly one-third of the protein and adopts a βαβαββα topology which contributes most of the contacts to the GSH substrate and is referred to as the GSH binding site (G-site). The C-terminal domain is all α-helical, is two-thirds of the protein and provides some of the contacts to the hydrophobic binding site (H-site) that lies adjacent to the GSH binding site. The H-site shows the greatest variability across GST classes and defines the substrate selectivities of the various isoenzymes [5, 8, 10]. The functional implication of the dimeric quaternary structure allows for the construction of a fully functional active site, part of which is situated near the subunit interface and is also a significant source of stabilization for the tertiary structures of the individual subunits.

There is evidence for at least five classes of insect GSTs based on their amino acid sequence homology, substrate specificity, physical properties and immunological properties. The insect class I GSTs or delta class are of interest because of their potential roles in insecticide resistance. The resistance to pesticides in insects has been shown to correlate with elevated levels of GST activity [2,11].

The *Anopheles dirus* mosquito *adgst1AS1* gene, obtained from a genomic library, is spliced to yield four mature GST transcripts; AdGSTD1, AdGSTD2, AdGSTD3 and AdGSTD4 [12]. This study focuses on AdGSTD4 only. AdGSTD4 has very high heterologous expression levels in *E. coli*. The high activity and catalytic efficiency toward GSH and CDNB substrates and the available crystal structure make this protein ideal for structure-function relationship studies [13,14].

This project aims to study the structure-function relationship of AdGSTD4 by characterizing critical residues that are in the active site. Leu33, His38 and His50 were investigated in this study. These residues are in the glutathione binding site (G-site) and His38 and His50 interact directly with the carboxylic group of glycine in GSH. These residues are conserved across insect delta class GSTs which suggests they play an important role. The selected residues were mutated and characterized for enzymatic properties as well as physical properties and compared to the AdGSTD4 wild type enzyme. The mutations showed effects not only in the kinetic properties and substrate specificity but also in the stabilities of the enzymes.

MATERIALS AND METHODS

Site Directed Mutagenesis

The mutants were generated using Stratagene's QuickChange Site directed mutagenesis protocol. The selected residues Leu33, His38 and His50 are in the G site. His38 and His50 directly interact with GSH. The mutagenic primers used in this experiment have been designed according to the 5' and 3' sequence of the AdGSTD4 wild type gene (Genbank accession number AF273040). The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling by means of *Pfu* DNA polymerase, which replicates both plasmid strands with high fidelity. Each mutant was randomly screened by restriction digestion analysis. Mutant plasmids could be distinguished from the template by digestion with the restriction enzyme corresponding to the restriction recognition site introduced by the mutagenic primers. Then full length DNA sequencing in both directions was performed to confirm the mutant clones.

Protein Expression and Purification

After transformation of the mutant plasmids into *E. coli* BL21(DE3)pLysS, protein expression was performed. All the AdGSTD4 mutants clones and wild-type were expressed in 200 ml LB broth (containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol) and induced with 0.1 M IPTG for 3 hours at 37 °C. The pellets were collected and kept at -20°C until used. The expression levels of the protein were determined by SDS-PAGE. The recombinant AdGSTD4 mutants and wild type were purified by using either glutathione affinity chromatography (GSTrap) or by using cation exchange followed by hydrophobic interaction chromatography. The GSTrap was used according to manufacturers instructions (Amersham Biosciences). The cation exchanger chromatography employed a SP-XL column equilibrated with Buffer A which was 20 mM Phosphate buffer pH 7 containing 10 mM dithiothreitol (DTT). After the lysate was applied to the column, the column was washed with buffer A. Then the protein was eluted with a linear gradient from 80-500 mM NaCl in buffer A. The major amount of GST enzyme eluted in buffer A containing 250 mM NaCl. The eluted GST fractions were pooled and loaded to a phenyl Sepharose column which was equilibrated with buffer A containing with 2 M NaCl. Washing steps were performed by using stepwise decreases in salt concentration in buffer A. The GST enzyme was eluted from the phenyl Sepharose column by using Buffer A containing 20% ethylene glycol and 10 mM DTT. The purified enzymes were stored in 50% glycerol at -20 °C until used. The concentrations of the proteins were determined by Bio-Rad protein reagent (Bio-Rad) and the purity of the proteins was analyzed by SDS-PAGE.

GST Activity Determination

The GST activity was determined for all the AdGSTD4 mutants and compared with the wild type protein. The standard GST assay was performed in 0.1 M potassium phosphate buffer pH 6.5 in the presence of 3 mM CDNB and 10 mM GSH [13]. The rate of conjugation between GSH and CDNB was monitored by continuously measuring the change in absorbance at 340 nm for 1 minute using a SpectraMax 250 at 25-27 °C. The extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ was used to convert the absorbance to moles [15].

Kinetic Parameters Determination

The kinetic experiments were performed as previously described [13]. In brief, CDNB was chosen as electrophilic substrate for determination of V_{max}, K_m, k_{cat} and k_{cat}/K_m values. The kinetic parameters were determined by varying the CDNB concentration (0.031-3.0 mM) while GSH was held constant at a saturating concentration and by varying the GSH concentrations (0.25-20 mM) at a saturating concentration of CDNB. The initial rate of the enzymatic reaction was measured spectrophotometrically as described for the GST activity assay determination. The steady state kinetics followed Michaelis-Menten kinetics except where stated. The maximal velocity (V_{max}) and the Michaelis constant (K_m) were determined by non-linear regression software analysis. The catalytic constant (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) were calculated on an active-site basis using the subunit molecular mass of each enzyme. The kinetic parameters are the mean ± standard deviation for at least three independent experiments.

Substrate Specificities Determination

The specific activities toward several GST substrates were determined by spectrophotometer, as previously described [13]. All measurements were performed at 25-27 °C in 0.1 M potassium phosphate buffer pH 6.5 or pH 7.5. The GST activities were measured with glutathione and five hydrophobic substrates; 1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), p-nitrophenethyl bromide (PNPBr) and p-nitrobenzyl chloride (PNBC). Specific activities were calculated according to the molar extinction coefficient (ε) for each substrate. The extinction coefficients were used to convert the absorbance to moles are of 9.6 $mM^{-1}cm^{-1}$, 8.5 $mM^{-1}cm^{-1}$, 5.0 $mM^{-1}cm^{-1}$, 1.2 $mM^{-1}cm^{-1}$ and 1.9 $mM^{-1}cm^{-1}$, respectively [15].

Half-Life Determination

The enzymes 0.1 mg/ml in 0.1 M phosphate buffer pH 6.5 containing 1 mM EDTA and 5 mM DTT were incubated at 45°C for various times and then the activity was measured in the standard GST assay. The data were plotted as log percentage of remaining activity versus pre-incubation time. The half-life of the enzyme at 45°C was calculated from the slope of the plot using the equations: Slope = k/2.3, $k = 0.693/t_{1/2}$.

RESULTS AND DISCUSSIONS

Kinetic Determination

As shown in Figure 1, the three residues Leu33, His38 and His50 are in the G-site [14]. However, the steady state kinetics of these mutants revealed that the role of these residues and their involvement in the GSH binding and enzyme catalysis varies (Table 1). The K_m toward CDNB of Leu33, His38, and His50 mutants are comparable to the wild type. This clearly demonstrates the amino acid changes at these positions in the GSH binding site did not affect the affinity of the enzyme to bind with this hydrophobic substrate. Leu33 mutants showed changes in both catalytic rate and GSH binding, although this residue does not interact directly with GSH. The data suggests that the mutations at this position affect the neighboring residues that influence GSH orientation in the active site.

The amino acid changes at position His50 drastically decreased V_{max} and k_{cat}, demonstrating this is a critical residue that functions in enzyme catalysis as well as in GSH binding. His38 mutants also exhibit differences in V_{max} or k_{cat} and K_m toward GSH. Notably the affinity toward GSH is very affected by the His38 mutations. The His38E enzyme steady-state kinetics did not obey the usual hyperbolic rate equation. The data fitted well to a rate equation expressing positive cooperativity between the two GSH binding sites. This means that during GSH binding, the glutamic acid in this position induces an active site conformational change which increases the GSH binding affinity in the second active site. Sigmoidal velocity curves have been reported previously for residues that impact upon part of the G-site [16-18].

These data suggest that His50 contributes more to enzyme catalysis than His38A although both His38 and His50 interact with the glycine of GSH. However the ionic interaction of His38 is implicated to be more important in the GSH binding compared to the H-bonding of His50.

The mutations at Leu33, His38 and His50 abolished the functional groups that either directly bind to GSH or to the adjacent residues. These residues influence the GSH interaction via hydrogen

bonding or assist in proper positioning of GSH in the active site during association. In addition the active site residues are well packed and mutations would change the topography of the active site yielding differences in the orientation of the residues thereby causing effects in substrate binding and enzyme catalysis.

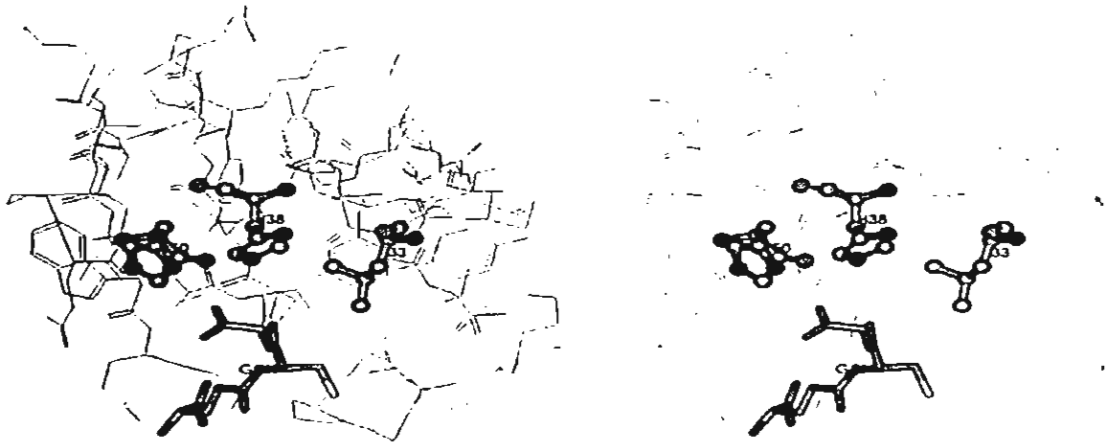


Figure 1. Stereoview of Leu33, His38 and His50 in the glutathione binding site. The coordinates for the tertiary structure are available from the Protein Data Bank with the accession number 1JLW.

Table 1. Kinetic parameters of the adGSTD4 wild type and mutant enzymes.

CLONE			CDNB		GSH	
	Vmax	kcat	Km	kcat/Km	Km	kcat/Km
	(μmol/min/mg)	(S^{-1})	(mM)	($sec^{-1}mM^{-1}$)	(mM)	($sec^{-1}mM^{-1}$)
Wild type	37.4 ± 1.4	15.60	0.74 ± 0.05	21.14	0.54 ± 0.07	28.96
L33A	23.5 ± 0.5	9.70	1.17 ± 0.05	8.30	8.20 ± 0.43	1.18
L33Y	0.32 ± 0.01	0.14	0.81 ± 0.12	0.17	1.10 ± 0.12	0.13
L33F	1.55 ± 0.03	0.65	1.30 ± 0.06	0.50	2.42 ± 0.22	0.27
H38A	50.0 ± 0.90	20.80	1.25 ± 0.12	16.64	15.8 ± 1.19	1.32
H38E	14.91 ± 0.44	6.23	0.92 ± 0.11	6.76	-*	-*
H38F	9.71 ± 0.43	4.06	0.98 ± 0.09	4.14	35.26 ± 0.86	0.12
H50A	8.3 ± 0.3	3.50	1.10 ± 0.15	3.18	10.9 ± 1.00	0.32
H50E	0.21 ± 0.01	0.09	0.80 ± 0.04	0.11	12.34 ± 0.36	0.01
H50Y	0.87 ± 0.02	0.36	0.83 ± 0.06	0.43	12.57 ± 0.73	0.03

*-Km cannot be determined, as the steady-state kinetics of this mutant does not obey the usual hyperbolic rate equation. $[S]_{0.5} = 7.74 \pm 0.12$ mM

Substrate Specificity

The substrate specificity data shows all three residue positions have influences on specificity (Table 2). The results show that the changes in the Leu33, His38 and His50 residues, which are in the GSH binding site, affected the ability of the enzyme to interact with a hydrophobic substrate. GSTs bind to GSH by an induced-fit mechanism [14, 19], therefore changing one amino acid in the active site may affect the topography of the enzyme binding pocket and the nearby residues that interact with both substrates.

Table 2. Substrate specificity determination using several GST substrates.

CLONE	Substrate Specificity (μmol/min/mg)				
	CDNB	DCNB	EA	PNPBr	PNBC
	(3 mM)	(1 mM)	(0.2 mM)	(0.1 mM)	(0.1 mM)
Wild-type	32.1 ± 1.2	0.035 ± 0.006	0.286 ± 0.062	0.074 ± 0.012	0.064 ± 0.002
L33A	18.3 ± 0.3	0.031 ± 0.015	0.332 ± 0.061	0.019 ± 0.004	0.024 ± 0.004
L33Y	0.253 ± 0.001	< 0.002	0.092 ± 0.006	< 0.006	< 0.007
L33F	1.023 ± 0.018	< 0.002	0.059 ± 0.007	< 0.006	< 0.007
H38A	21.7 ± 0.2	0.037 ± 0.001	0.146 ± 0.016	0.040 ± 0.004	0.013 ± 0.002
H38E	11.21 ± 0.24	< 0.001	0.191 ± 0.026	< 0.008	< 0.005
H38F	3.76 ± 0.09	< 0.001	0.230 ± 0.019	< 0.005	< 0.003
H50A	3.8 ± 0.1	0.015 ± 0.007	0.146 ± 0.050	0.012 ± 0.002	0.016 ± 0.007
H50E	0.149 ± 0.004	< 0.001	0.026 ± 0.004	< 0.005	< 0.005
H50Y	0.466 ± 0.011	< 0.001	0.064 ± 0.008	< 0.008	< 0.005

The active site pocket of GSTs are composed of a GSH binding site and a hydrophobic substrate binding site. Although the residues in the present study are in the GSH binding site, there are differences in the substrate specificity for several compounds. It has been suggested that mutations of active site amino acids affects the whole electrostatic field in the active site. The electrostatic field in the active site involves many amino acids that influence atom movement, active site packing, charge distribution, GSH ionization, and GSH orientation to a suitable position for conjugation with the electrophilic GST substrates [20-22]. If the electrostatic field is affected, it will affect the charge distribution and the topography of the active site thereby altering the substrate conjugation ability of the enzyme.

Half-Life Determination

The half-life of the enzymes at 45°C compared to the AdGSTD4 wild-type showed remarkable differences in the Leu33 mutants (Table 3). However the His38 and His50 mutant enzymes displayed only small increases in stability suggesting a minimal role in structural maintenance. The crystal structure shows Leu33 and its neighboring residues Leu6, Thr31 and Ile52 form part of the wall in the GSH binding

site. Therefore the Leu33 residue is involved in the packing of the active site which also impacts upon the tertiary structure of the whole protein.

Table 3. Half life determination at 45 °C.

CLONE	Half-life at 45 °C (Min)
Wild-type	15.32 ± 0.31
L33A	45.29 ± 1.56
L33Y	71.65 ± 1.71
L33F	211.73 ± 17.90
H38A	15.33 ± 0.88
H38E	40.17 ± 1.26
H38F	19.33 ± 0.59
H50A	25.81 ± 1.99
H50E	15.31 ± 0.54
H50F	20.79 ± 0.34

CONCLUSIONS

The present study investigated three residue positions in the glutathione binding site in proximity to interact with the glycine moiety of the glutathione substrate. Each of these residues appeared to have unique and multiple roles in the enzyme including contributions to substrate binding, the multiple steps of catalysis as well as influencing substrate specificity. Some of these functions appear to originate or are modified by the changes in the topography of the active site, for example Leu33 did not appear to directly interact with glutathione but was involved with formation of the active site wall. The changes in the topography or conformation of the active site also appeared capable of exerting major influences on tertiary structural organization of the whole GST protein such that a 13-fold increase in enzyme stability was observed for one Leu33 mutant. These results demonstrate that residues in the active site are not only important in the catalytic function but also may play a role in the structural integrity of the enzyme.

ACKNOWLEDGEMENTS

This work was funded by the Thailand Research Fund (TRF). A.V. was supported by a Royal Golden Jubilee Scholarship.

REFERENCES

[1] Mannervik, B., (1985) *Adv. Enzymol. Relat. Areas Mol. Biol., 57*, 357-417.
[2] Hayes, J.D. and Pulford, D.J., (1995) *CRC Crit. Rev. Biochem. Molec. Biol., 30*, 445-600.

[3] Mannervik, B. and Danielson, U.H., **(1988)** *CRC Crit. Rev. Biochem., 23,* 283-337.
[4] Jakoby, W. B., Habig, W. H. in *Enzymatic Basis of Detoxication Vol. 2*Ed.: W. B. Jakoby), Academic Press, New York **1980**, p. pp. 63-94.
[5] Armstrong, R.N., **(1997)** *Chem. Res. Toxicol., 10,* 2-18.
[6] Caccuri, A.M., Ascenzi, P., Antonini, G., Parker, M.W., Oakley, A.J., Chiessi, E., Nuccetelli, M., Battistoni, A., Bellizia, A. and Ricci, G., **(1996)** *J. Biol. Chem., 271,* 16193-16198.
[7] Armstrong, R.N., Rife, C. and Wang, Z., **(2001)** *Chemico-Biological Interactions, 133,* 167-169.
[8] Dirr, H., Reinemer, P. and Huber, R., **(1994)** *Eur. J. Biochem., 220,* 645-661.
[9] Ketterer, B., **(2001)** *Chemico-Biological Interactions, 138,* 27-42.
[10] Wilce, M.C.J. and Parker, M.W., **(1994)** *Biochim. Biophys. Acta, 1205,* 1-18.
[11] Prapanthadara, L., Hemingway, J. and Ketterman, A.J., **(1993)** *Pestic. Biochem. Physiol., 47,* 119-133.
[12] Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L. and Ketterman, A.J., **(2001)** *Insect Biochem. Molec. Biol., 31,* 75-85.
[13] Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L. and Ketterman, A.J., **(2001)** *Insect Biochem. Molec. Biol., 31,* 867-875.
[14] Oakley, A.J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A.J. and Wilce, M.C.J., **(2001)** *Protein Science, 10,* 2176-2185.
[15] Habig, W.H., Pabst, M.J. and Jakoby, W.B., **(1974)** *J. Biol. Chem., 249,* 7130-7139.
[16] Stenberg, G., Abdalla, A. M. and Mannervik, B., **(2000)** *Biochem. Biophys. Res. Comm., 271,* 59-63.
[17] Ricci, G., Caccuri, A.M., Lo Bello, M., Rosato, N., Mei, G., Nicotra, M., Chiessi, E., Mazzetti, A.P. and Federici, G., **(1996)** *J. Biol. Chem., 271,* 16187-16192.
[18] Ricci, G., Lo Bello, M., Caccuri, A.M., Pastore, A., Nuccetelli, M., Parker, M.W. and Federici, G., **(1995)** *J. Biol. Chem., 270,* 1243-1248.
[19] Oakley, A.J., Lo Bello, M., Ricci, G., Federici, G. and Parker, M.W., **(1998)** *Biochemistry, 37,* 9912-9917.
[20] Stevens, J.M., Armstrong, R.N. and Dirr, H.W., **(2000)** *Biochem. J., 347,* 193-197.
[21] Labrou, N.E., Mello, L.V. and Clonis, Y.D., **(2001)** *Biochem. J., 358,* 101-110.
[22] Chuang, C. C., Wu, S. H., Chiou, S. H. and Chang, G. G., **(1999)** *Biophys. J., 76,* 679-690.

Received on June 17, 2003, accepted on July 23, 2003.

Protein and Peptide Letters, Vol. 10, No. 4, pp. 375-385, 2003
Bentham Science Publishers Ltd.
0929-8665/03 $ 41.00 + .00

A NON-ACTIVE SITE RESIDUE, CYSTEINE 69, OF GLUTATHIONE S-TRANSFERASE ADGSTD3-3 HAS A ROLE IN STABILITY AND CATALYTIC FUNCTION.

Jeerang Wongtrakul, Issara Sramala and Albert J. Ketterman*

Institute of Molecular Biology and Genetics, Mahidol University Salaya Campus, Salaya, Nakhon Pathom 73170, Thailand
*Email: frakt@mahidol.ac.th

ABSTRACT: The Cys69 residue of an *Anopheles dirus* glutathione S-transferase isoform (adGSTD3-3), was characterized to elucidate its contribution in both catalysis and structural support. Nine mutants were generated at this position by replacing the residue with polar, non-polar and charged residues. The polar residues changed the V_m of the enzymes. With non-polar residues, the enzymes were unable to fold and were expressed in the insoluble inclusion form. With charged residues, the soluble enzyme yields were only 3% of the wild type protein. Molecular dynamics simulation also was performed to understand the changes in the enzyme structure. These findings are additional evidence of the importance of structural residues that affect the enzymatic properties such as V_m, K_m and enzyme specificity.

Keywords: Glutathione transferase, *Anopheles dirus*, mosquito, structure, mutagenesis, catalysis.

INTRODUCTION

Glutathione S-transferases (GSTs; EC. 2.5.1.18) are dimeric multifunctional enzymes that have a major role in detoxication of physiological substances as well as xenobiotic compounds such as nitric oxide, drugs, herbicides and insecticides [1, 2]. The enzymes function by catalyzing the nucleophilic addition of the thiol group of reduced glutathione to electrophilic centers in organic compounds [3]. They also have a ligand-binding function using either the hydrophobic substrate-binding site or the cleft located along the dimer interface [4-6]. Recently identified functions of GSTs include roles in signal transduction through inhibition of Jun-N terminal kinase (JNK) and Apoptosis signal-regulating kinase 1 (ASK1) [7, 8]. GSTs have been grouped into distinct classes on the basis of sequence and structural homology, immunochemistry, and substrate specificity. There are at least 12 classes of cytosolic GST; alpha, mu, pi, sigma, theta, kappa, omega, zeta, beta, phi, tau and delta [9, 10].

All GST molecules are composed of two subunits, each containing two domains, an N-terminal and a C-terminal domain that provide two functional active sites per native dimer. Each active site can be divided into subsites that bind the glutathione (the G-site) and the hydrophobic electrophilic substrate (the H-site). The G-site is formed by the N-terminal domain, which is structurally related to thioredoxin. The H-site is formed by the C-terminal region. The G-site is well conserved but the H-site is highly variable and contributes to the varying substrate specificities of the different isoenzymes [11-16]. The interaction at the subunit interface of GSTs in sigma, theta and delta classes are predominantly hydrophilic compared with the more hydrophobic nature of the interfaces of the alpha, mu and pi class GSTs [17-23].

Based on many GST tertiary structures, there are only a few cysteine residues found in each subunit. In pi class, there are four cysteine residues at position 14, 47, 101 and 169 from the amino terminus. Although it is not a predominant amino acid it was shown to have an important role in both catalysis and structural support contributed by disulfide bonds. The intrasubunit disulfide bond is formed by the interaction of Cys47 and Cys101 whereas the intersubunit disulfide bond is formed between Cys47 residues in different subunits [24]. The carboxymethylation of cysteine at position 47 increased the K_m for glutathione by three orders of magnitude [25]. In addition, site-directed mutagenesis of four cysteine residues in pi class revealed that Cys14 was involved in the catalysis by stabilizing the structure of an active site loop. A Cys47Ser mutation decreased the binding affinity toward glutathione. In contrast the substitution of Cys101Ser increased the binding affinity of CDNB substrate by changing the conformation of helix 4. The Cys169 residue appeared to have an important role for maintaining a stable conformation of the enzyme [26].

The insect delta class GSTs are thought to have a role in insecticide resistance. Thus understanding structure-function relationships of amino acids in insect GSTs is necessary to identify important residues that affect the enzymatic properties. The adGSTD3-3 isoform, one of four splicing products of adGST1AS1 gene [27], contains three cysteine residues per subunit, Cys12, Cys69 and Cys106. Cys12 and 106 are conserved among all four isoforms whereas Cys69 is found only in adGSTD3-3. The Cys69 residue, located at the N-terminus of helix 3 in domain I, was one out of five residues chosen in a previous study. The replacement of Cys69 with glutamine, the residue of the adGSTD1-1 isoform, significantly increased the V_m of the enzyme approximately 65%. Therefore this work aimed to continue the characterization of Cys69 by replacing the residue with different amino acids.

Methods

Site-directed mutagenesis

The plasmid pET3a containing adGSTD3-3 coding sequence has previously been constructed [28]. The construct was used as a DNA template in a PCR reaction according to Quick Change™ site-directed mutagenesis described by Stratagene. The following oligonucleotides C69Q-f: 5'GCGCGCCATCCA GACGTACTTAGCGGAGAAGTACGGCAAG3', C69E-f:5'GAGTCGCGCGCCATCGAGACGTACCTGG CGGAGAAGTAC3', C69F-f: 5'GAGTCGCGCGCCATCTTCACGTACCTGGCGGAGAAGTAC 3', C69K-f: 5'GAGTCGCGCGCCATCAAAACGTACCTGGCGGAGAAGTAC 3', C69W-f: 5'GAGTCGCGCGCCAT

CTGGACGTACCTGGCGGAGAAGTAC 3', C69Y-f: 5' GAGTCGCGCGCCATCTATACGTACTTGGCG GAGAAG 3', C69P-f: 5'CTGTGGGAGTCGCGCGCCATCCCTACGTACTTGGCGGAG 3', C69A-f: 5' GA GTCGCGCGCCATCGCTACGTACTTGGCGGAGAAG 3', C69N-f: 5' CTGTGGGAGTCGCGCGCCATC AATACGTACTTGGCGGAG 3' were used as mutagenic primers for the Cys69 mutants. The changed nuclotide or amino acid residues are shown in bold type and the additional recognition site for restriction endonuclease are underlined. The recombinant plasmids were randomly screened by restriction analysis then the nucleotide sequences of the plasmids carrying the mutations were verified by full-length sequencing in both directions using a Bigdye™ Terminator Cycle Sequencing Kit (Perkin Elmer).

Protein expression and purification

The protein expression and purification were carried out as previously described [29]. After affinity purification, the wild type and Cys69 mutant enzymes were homogeneous as judged by SDS-PAGE. The protein concentration was determined by the method of [30] using Bradford protein reagent with bovine serum albumin as the standard protein.

Kinetic studies

The GST activity assays were performed as previously described [31]. Thermal stability was measured as a function of time. The different Cys69 mutants were incubated (0.1 mg/ml in 0.1 M potassium phosphate pH 6.5 containing 5 mM DTT and 1 mM EDTA) at 45 °C and aliquots were assayed for activity in the standard system at different time-points. Half-lives for the enzymes represent the time of incubation when there is 50% residual activity and were calculated using the equations: Slope = k/2.3, $k = 0.693/t_{1/2}$. The slope was obtained from the linear plot between log percentage of original activity and the time point of preincubation.

Molecular dynamics simulation

Several one-nanosecond molecular dynamics trajectories of adGSTD3-3 in complex with glutathione was generated. Gromac 3.0 (http://www.gromacs.org) with Gromacs force fields was used throughout the study [32, 33]. The same adGSTD3-3 coordinates were used as the initial structure for the simulation. Residue Cys69 was replaced with glutamine and asparagine residues using Deepview Swiss-PdbViewer software (http://www.expasy.org/spdbv/)[34]. The file was then converted into (.GRO) format. The protein was immersed in a cubic box full of water molecules. Na^+ ions sufficient to neutralize the systems were added by replacing some of the water molecules to minimize the protein-ion electrostatic interaction. A steepest descent energy minimization was performed followed by positional restraint to fix the protein structure and allow interaction with water molecules. The simulation was carried out for 1 ns. The crystal structure and simulated structures were visualized either by DS ViewerPro 5.0 (Accelrys Inc) or Deepview Swiss-PdbViewer v3.5b4.

Figure 1. The stereoview of domain 1 of adGSTD3-3 isoform crystallized with glutathione. Three active site residues in helix 3; Glu64, Ser65 and Arg66, glutathione and Cys69 residue are shown using ball and stick representation. This figure was created with DS ViewerPro 5.0.

RESULTS

Expression and purification

To understand the role of Cys69 in both catalysis and structure, nine mutants at this position were generated using PCR based site-directed mutagenesis. The Cys was replaced with amino acids of different properties; polar, non-polar and charged. The location of Cys69 is shown in Figure 1. Cys69Gln, Cys69Ala and Cys69Asn mutants were expressed in *E. coli* at 37°C with a band size of approximately 23 kDa, but the yields of soluble protein varied for the different mutant enzymes. The wild type enzyme and mutant Cys69Gln yielded approximately 30-40% of total soluble protein from the bacterial lysate. For the Cys69Ala and Cys69Asn mutants this value was 17% and 10% respectively. There were six Cys69 mutants, Cys69Pro, Cys69Phe, Cys69Lys, Cys69Glu, Cys69Trp and Cys69Tyr that expressed GST in an inclusion form. Therefore the expression temperature was lowered to 25°C or 18°C. At 25°C, only Cys69Glu was soluble. However, the GST was expressed at a relatively low level approximately 3% of the total protein in the lysate. The majority of the protein expressed as inclusion body. Cys69Phe and Cys69Lys had detectable activity in the lysate however the protein concentration obtained after the purification was approximately 0.02-0.03 mg/ml. The specific activity of both mutants was therefore calculated to be 12.9 and 1.3 μmol/min/mg. The actual activity is less since the SDS-PAGE showed contaminating bands indicating that the enzymes were not absolutely pure. The remaining Cys69 mutants, Cys69Pro, Cys69Trp and Cys69Tyr, were all expressed as inclusion bodies at both 25°C and 18°C.

Table 1. Michaelis-Menten parameters for adGSTD3-3 and Cys69 mutants.

Enzyme	V_m	k_{cat}	GSH		CDNB	
			K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
AdGSTD3-3	63.6 ± 6.2	25.2	0.29 ± 0.03	86.9	0.13 ± 0.01	194
Cys69Gln	105 ± 7	41.7	0.44 ± 0.03	96.9	0.21 ± 0.03	198
Cys69Asn	26.7 ± 6.4	9.61	0.53 ± 0.03	18.2	0.08 ± 0.01	117
Cys69Ala	65.5 ± 7.5	25.9	0.33 ± 0.04	79.2	0.11 ± 0.03	235
Cys69Glu	57.1 ± 1.6	22.6	0.53 ± 0.03	42.6	0.13 ± 0.01	173

All measurements were performed at 25-27°C as described under Methods. The k_{cat}/K_m values were calculated from the initial linear plot of the saturation curve at low concentrations of the varied substrates.

Table 2. Specific activity of the Cys69 mutants towards five different substrates.

Enzyme	CDNB (1 mM)	DCNB (1 mM)	4-nitrobenzyl chloride (0.1 mM)	4-nitrophen-ethyl bromide (0.1 mM)	Ethacrynic Acid (0.2mM)
AdGSTD3-3	66.6 ± 1.8	0.326 ± 0.007	0.099 ± 0.006	0.003 ± 0.002	0.093 ± 0.005
Cys69Gln	94.6 ± 3.2	0.259 ± 0.014	0.132 ± 0.010	0.018 ± 0.002	0.143 ± 0.008
Cys69Asn	40.9 ± 2.0	0.350 ± 0.007	0.161 ± 0.020	0.029 ± 0.006	0.146 ± 0.019
Cys69Ala	54.2 ± 3.5	0.217 ± 0.002	0.131 ± 0.014	0.030 ± 0.009	0.163 ± 0.001
Cys69Glu	63.6 ± 3.8	0.234 ± 0.016	0.184 ± 0.019	0.028 ± 0.009	0.345 ± 0.056

The units are μmole/min/mg of protein. The data are mean ± standard deviation from at least 3 independent experiments.

Catalytic properties

The catalytic properties of adGSTD3-3 and Cys69 mutants are summarized in Table 1. It is shown that the residue in position 69 has a strong influence on kinetic parameters investigated. The effect is greatest for the Cys69Gln and Cys69Asn mutants. Cys69Gln possessed a V_m more than 1.6-fold of the wild type whereas Cys69Asn is approximately 2.4-fold less than the wild type. The change of Cys69 with asparagine significantly decreased the k_{cat}/K_m^{GSH} 4.8-fold compared to the wild type adGSTD3-3. The effect results from a combination of reduced k_{cat} and increased K_m^{GSH} values. For the Cys69Glu mutant on the other hand, a significantly increased K_m^{GSH} is the cause for the lower catalytic efficiency. Although the replacement of Cys69 with asparagine increased the binding affinity toward CDNB substrate the k_{cat}/K_m^{CDNB} is significantly lower, approximately 1.6 fold due to the low k_{cat} value. Kinetic constants for the Cys69 mutants strongly suggest that the amino acid at position 69 has a major structural influence on the catalytic activity of the enzyme. The specific activities of the purified enzymes were determined with five different substrates (Table 2). Replacing the cysteine in position 69 by alanine or asparagine reduced the specific activity with CDNB approximately 1.2-1.6-fold whereas the replacement by glutamine significantly increased the activity about 1.4-fold. Specific activities determined with DCNB, a mu class substrate, were lower for the mutant Cys69Gln, Cys69Ala and Cys69Glu approximately 1.2-1.5-fold compared to the wild type. Greater specific activities toward 4-nitrobenzyl chloride, 4-nitrophenethyl bromide and ethacrynic acid were observed with all the Cys69 mutants. Cys69Glu showed the highest conjugating activity towards 4-nitrobenzyl chloride and ethacrynic acid compared to the other Cys69 mutants or the wild type enzymes. An inhibition study of CDNB activity was performed using several

different compounds that represent hydrophobic substrates or glutathione analogs including pyrethroid insecticides (Table 3). The inhibition of Cys69Gln with 4-nitrobenzyl chloride and 4-nitrophenethyl bromide was less than for the wild type, approximately 1.3 and 1.5 fold respectively. With the pyrethroid insecticide deltamethrin, Cys69Gln and Cys69Ala had approximately 1.5 fold more inhibition than the wild type. Cys69Asn demonstrated the lowest inhibition toward cumene hydroperoxide and ethacrynic acid compared to the wild type and the other mutants. Based on the results obtained from Tables 2 and 3, it is suggested that the replacement of amino acid 69 in domain I affected the binding of both glutathione and hydrophobic substrates since the enzymes possessed different conjugating activities and inhibition characteristics.

Table 3. Percent inhibition study of adGSTD3-3.

Inhibitor	Wild type	Cys69Gln	Cys69Asn	Cys69Ala	Cys69Glu
4-NBC 1mM	49 6 ± 4 9	37 4 ± 1 6	55 0 ± 4 9	55 2 ± 2 1	46 4 ± 1 9
CuHOOH 2 5 mM	26 9 ± 2 5	25 7 ± 0 2	17 5 ± 0 9	26 6 ± 4 5	27 8 ± 1 4
DCNB 1 mM	46 0 ± 3 1	49 9 ± 4 2	46 6 ± 4 0	51 6 ± 3 6	43 1 ± 2 0
4-NPB 0.1 mM	27 8 ± 3 1	18 2 ± 2 1	36 5 ± 0 6	33 4 ± 1 9	26 2 ± 3 7
Deltamethrin 0 01 mM	47 7 ± 5 9	70 0 ± 4 2	44 4 ± 2 4	68 3 ± 1 5	46 7 ± 1 1
Permethrin 0 01 mM	38 9 ± 6 7	59 8 ± 2 7	32 4 ± 2 6	52 8 ± 7 9	39 1 ± 5 9
EA 0 1 mM	96 0 ± 1 2	96 6 ± 1 8	96 9 ± 0 8	96 0 ± 2 0	95 5 ± 0 5
EA 0 01 mM	74 6 ± 1 9	77 1 ± 1 1	74 5 ± 1 6	79 4 ± 4 2	73 7 ± 3 2
EA 0 001 mM	27 6 ± 2 1	25 0 ± 7 9	17 8 ± 3 7	37 0 ± 4 8	41 0 ± 1 5
S-hexyl 0.1 mM	63 5 ± 2 9	71 7 ± 1 4	58 7 ± 3 7	65 1 ± 2 9	54 1 ± 2 9
S-hexyl 0 01 mM	20 3 ± 3 6	24 1 ± 4 5	15 2 ± 2 5	13 1 ± 2 4	33 4 ± 4 9

The data are means ± standard deviation from at least three separate experiments. The tested compounds are 4-NBC, 4-nitrobenzyl chloride, CuHOOH, Cumene hydroperoxide, DCNB, 1,2-dichloro-4-nitrobenzene, 4-NPB, 4-nitrophenethyl bromide, deltamethrin, permethrin, EA, ethacrynic acid, S-hexyl, S-hexylglutathione. The activity was measured using the standard GST assay

Stability assay

The nature of the side-chain in position 69 is of importance for thermal stability as shown in Table 4. Removal of the sulfhydryl group of Cys69 gives mutant Cys69Ala a significantly less stable enzyme with a 2.5-fold decrease in half-life. The replacement of cysteine with a negatively charged amino acid, Cys69Glu, reduces the thermal stability 3.8-fold. The replacement of the sulfhydryl group with a larger polar amino acid, Cys69Gln, reduces the stability 1.4-fold. On the other hand, the replacement with a smaller hydrophilic R group Asn, one methylene group different, significantly decreased the stability of the enzyme approximately 5.4 fold. The 69 residue is also involved in the packing of the hydrophobic core in domain I since the replacement of Cys69 with Phe, Trp, Pro, Lys and Tyr which consist of larger side chains compared to Gln, caused the protein to be expressed in an inclusion form. Therefore this residue position appeared to have a role in both folding and stability of adGSTD3-3.

Molecular dynamics simulation

To explain the changes in enzymatic activity including the stability of the Cys69 mutants, models of Cys69Gln and Cys69Asn were generated and dynamics simulations were conducted for 1000

Table 4. Thermal stability of GST mutants.

Enzymes	Half-life (minutes)
Wild type	3.8 ± 0.03
Cys69Gln	2.8 ± 0.16
Cys69Asn	0.7 ± 0.08
Cys69Ala	1.5 ± 0.25
Cys69Glu	1.0 ± 0.06

Half-lives represent the time of incubation after which 50% activity remains measured in the standard assay system, the conjugation between CDNB (1 mM) and GSH (10 mM).

Figure 2. Comparison of the wild type adGSTD3-3 (blue) and Cys69Asn (yellow) conformations obtained from the molecular dynamics trajectories. Shown is the superimposition using the time point that demonstrated maximum changes in the center of gravity at 1000 ps. Both 69 residues are shown in stick form, Cys69 (red) and Asn69 (green).

picoseconds (ps). The variations in the system were also determined by performing four simulations of the wild type for 200 ps each using the structure obtained from 1000 ps as the starting coordinates and four different random seed numbers in the system. The parameters were analyzed during the 500-1000 ps duration. During this period the structure reaches a conformational fluctuation steady state, with similar values to the average values of the wild type models obtained from 200 ps, e.g. hydrogen bonds, radius of gyration, RMSD and dihedral angle. In addition, Root Mean Square Fluctuation (RMSF) was also computed for each residue to illustrate the fluctuation pattern within the protein. The RMSF pattern showed that residues 24-41, 77-87, 119-124, 142-155 and 190-193 are the flexible regions of the protein. These residues are in the loops between helices except 24-41, which also includes β2. The helix 2 in pi class was shown to undergo the most prominent conformational changes in a dynamics simulation [35]. No appreciable change of the monitored structural parameters takes place relative to the structure of the wild type at 1000 ps, except for the location of Cys69 relative to the Center of gravity (Cg) that indicates a different conformation. A snapshot of that time point in both mutants was generated and superimposed on the wild type. It was found that helix 3 was shifted in both Cys69Asn and Cys69Gln approximately 1.4 and 1.7 Å, measured between C-alpha of residue 69 in wild type and mutant (Figure 2). The most relevant conformational changes are observed in the structure comprising residues 32-41, the loop between β2 and α2 (maximum deviation 4 Å) and residues 77-87, the loop before helix 4 (maximum deviation 5 Å) that correlates with the RMSF parameters.

DISCUSSION

The Cys69 position appears to have an impact on a sensitive structural region of the adGSTD3-3 isoform by changing both catalysis and stability of the enzyme. The tertiary structure of adGSTD3-3 shows the Cys69 residue relatively distant from the other two cysteine residues. Therefore, it is unlikely that the residues could form disulfide bonds to each other to stabilize the structure. It was found that the Cys69

mutants that possessed larger side chains such as Trp, Tyr and Phe were unable to fold into the appropriate structures and were expressed in the inclusion form, indicating severe structural perturbations. Although it had no effect on the kinetic parameters, the smaller side chain alanine reduced the enzyme half-life more than 50%. In addition, when residue 69 was replaced with either a negatively charged amino acid (Glu) or a positively charged residue (Lys), both mutants were expressed as inclusions at 37°C but only the Cys69Glu could express as soluble protein at a lower temperature. However, the soluble GST form obtained was only 3% because the majority of the expression was still in an inclusion form. This was also found in two temperature-sensitive mutants of GST pi, Asp153Ala and Ser150Ala/Asp153Ala mutants [36]. The expression of both mutants depended on the temperature of host cell growth. The yield of the enzymes was lower and clearly decreased with increasing temperatures. All these results strongly suggest that residue 69 is involved in the enzyme folding as well as stability since all Cys69 mutants demonstrated dramatically decreased half-lives. However the relationship between kinetic parameters including the thermal stability of the enzyme and the property of the amino acid at position 69 remains unclear.

The adGST Cys69 mutation also may affect residues that maintain an intersubunit salt bridge of the protein. The crystal structure shows Glu74 in helix 3 in domain 1, forms an intersubunit salt bridge with Arg90 in helix 4 in domain 2 of the neighboring subunit, to stabilize the structure. The distances of both residues measured in Cys69Gln and Cys69Asn were 5.43 and 4.86 Å therefore the replacement of Cys69 residue would cause both mutants to lose their intersubunit salt bridges. A salt bridge interaction was also found to stabilize GST structure in a rat mu class GST [37]. Equilibrium folding studies of insect GSTs will increase our understanding of the roles played by individual domains in maintaining the stability of GST dimers and their monomers. GST folding is thought to originate by the formation of three dimensional structural formations (nucleation) occurring independently in the individual domains. Based on the structure, domain 1 (approximately residues 1-80) adopts a topology similar to that of the thioredoxin fold consisting of four β-sheets with three flanking α-helices. The folding in this domain is initiated by interaction between β2 and β4 regions [38]. Domain 2 (approximately residues 81-207) consists of two important motifs, an N-capping box and a hydrophobic staple motif, located at the beginning of helix 6. The N-capping box has an important role in folding and stability in GSTs by determining the direction of the forming helix [36]. The latter motif is required in the cooperativity between α6 helix in domain 2 and α1 in domain 1 [39, 40]. Both motifs have been proposed as nucleation sites of folding. Moreover, helix 3 was also found to have a critical role in maintaining the correct folding of domain 2 [41, 42].

The interaction at the subunit interface also has a major effect on the folding in GST. It also may have a role in determining GST stability. Based on GST tertiary structures, the equilibrium unfolding of a homodimeric delta class GST should be most closely related to the sigma and theta class since all three structures have hydrophilic interactions at the subunit interface. A four-state pathway for a sigma class GST was proposed with triphasic unfolding transition [43]. The first transition is protein concentration independent and involves a change in the subunit tertiary structure producing a partially active dimeric intermediate ($N_2 \leftrightarrow I_2$). The second step is protein concentration dependent with I_2 dissociation into two

monomeric intermediates. The final step of unfolding transition is protein concentration independent and involves the complete unfolding to the monomeric state. Although having a lock-and-key intersubunit interaction, the unfolding of alpha and pi class GSTs are two-state pathways [44, 45] but the equilibrium unfolding/refolding of class mu proceeds via a three-state process [46].

The replacement of Cys69 significantly affected the kinetic parameters especially the V_m, K_m^{GSH} and k_{cat}/K_m for GSH (Table 1) suggesting that the conformation of the G-site was altered. Cys69 is located at the N-terminus of α3-helix in domain 1 adjacent to residues Glu64, Ser65 and Arg66, active site residues that directly interact with the glutamate residue of glutathione. Gln64 in GSTPi, the equivalent residue of our Glu64, was shown after mutation to alanine to significantly decrease the yield of protein expression and glutathione-binding affinity including the specific activity [47, 48]. Static models also provided a clue of subtle conformational changes of adGSTD3-3 by detecting the atom movement between residues 69 and 65. The movement may also induce the positional change of Arg66 since Ser65 forms an H-bond with Arg66, which forms a salt bridge with Asp100 and appears to stabilize the intra-subunit domains of the protein. Based on the superimposition of wild type, Cys69Gln and Cys69Asn obtained from dynamics simulation, the most conspicuous conformational changes are observed in helix 3. The whole helices of both mutant models were shifted to a greater extent than the wild type structure at the C-terminal region. Other regions such as residues 32-41 in the loop before helix 2, and residues 77-87 in the loop before helix 4 also demonstrated relevant structural rearrangement with maximum deviations of 4-5 Å. These also provided additional evidence of conformational changes. The effect of the structural changes may cause a major change of the catalytic property of adGSTD3-3 since some regions e.g. the loop before helix 2 contains several residues in the active site region of adGSTD3-3. Indeed, this has been shown in other GSTs to control the movement of helix 2 that participates in the formation of active site and modulates the G-site affinity for glutathione [49].

In conclusion, these results strongly suggest that residue 69 is involved in the initial enzyme folding as well as stability since all Cys69 mutants have dramatically decreased half-lives and several mutants could only be expressed as insoluble inclusion bodies. These findings are additional evidence of important structural residues that affect the enzymatic properties such as V_m, K_m and enzyme specificity.

ACKNOWLEDGEMENTS

This work was funded by the Thailand Research Fund (TRF) and a Royal Golden Jubilee Grant. J.W. was supported by a Royal Golden Jubilee Scholarship.

REFERENCES

[1] Hayes, J. D., Wolf, C. R. In *Glutathione Conjugation. Mechanisms and biological significance.* Eds.: Sies, H., Ketterer, B., Academic Press, London **(1988)**, pp. 315.
[2] Lo Bello, M., Nuccetelli, M., Caccuri, A.M., Stella, L., Parker, M.W., Rossjohn, J., McKinstry, W.J., Mozzi, A.F., Federici, G., Polizio, F., Pedersen, J.Z. and Ricci, G. **(2001)** *J.Biol.Chem., 276,* 42138.
[3] Booth, J., Boyland, E. and Sims, P. **(1961)** *Biochem.J., 79,* 516.
[4] Lyon, R.P. and Atkins, W.M. **(2002)** *Biochemistry, 41,* 10920.
[5] Oakley, A.J., Lo Bello, M., Nuccetelli, M., Mazzetti, A.P. and Parker, M.W. **(1999)** *J.Mol.Biol., 291,* 913.

[6] Sayed, Y., Wallace, L.A. and Dirr, H.W. (2000) *FEBS Lett., 465,* 169.
[7] Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J. and Ronai, Z. (1999) *EMBO J., 18,* 1321.
[8] Cho, S.-G., Lee, Y.H., Park, H.-S., Ryoo, K., Kang, K.W., Park, J., Eom, S.-J., Kim, M.J., Chang, T.-S., Choi, S.-Y., Shim, J., Kim, Y., Dong, M.-S., Lee, M.-J., Kim, S.G., Ichijo, H. and Choi, E.-J. (2001) *J.Biol.Chem., 276,* 12749.
[9] Armstrong, R.N. (1997) *Chem.Res.Toxicol., 10,* 2.
[10] Sheehan, D., Meade, G., Foley, V.M. and Dowd, C.A. (2001) *Biochem.J., 360,* 1.
[11] Bruns, C.M., Hubatsch, I., Ridderström, M., Mannervik, B. and Tainer, J.A. (1999) *J.Mol.Biol., 288,* 427.
[12] Dirr, H., Reinemer, P. and Huber, R. (1994) *Eur.J.Biochem., 220,* 645.
[13] García-Sáez, I., Párraga, A., Phillips, M.F., Mantle, T.J. and Coll, M. (1994) *J.Mol.Biol., 237,* 298.
[14] Ji, X., Zhang, P., Armstrong, R.N. and Gilliland, G.L. (1992) *Biochemistry, 31,* 10169.
[15] Oakley, A.J., Rossjohn, J., Lo Bello, M., Caccuri, A.M., Federici, G. and Parker, M.W. (1997) *Biochemistry, 36,* 576.
[16] Reinemer, P., Dirr, H.W., Ladenstein, R., Schäffer, J., Gallay, O. and Huber, R. (1991) *EMBO J., 10,* 1997.
[17] Ji, X., Von Rosenvinge, E.C., Johnson, W.W., Tomarev, S.I., Paitigorsky, J., Armstrong, R.N. and Gilliland, G.L. (1995) *Biochemistry, 34,* 5317.
[18] Oakley, A.J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A.J. and Wilce, M.C.J. (2001) *Protein Science, 10,* 2176.
[19] Reinemer, P., Dirr, H.W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G. and Parker, M.W. (1992) *J.Mol.Biol., 227,* 214.
[20] Reinemer, P., Prade, L., Hof, P., Neuefeind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Barrunik, H.D. and Bieseler, B. (1996) *J.Mol.Biol., 255,* 289.
[21] Sinning, I., Kleywegt, G.J., Cowan, S.W., Reinemer, P., Dirr, H.W., Huber, R., Gilliland, G.L., Armstrong, R.N., Ji, X., Board, P.G., Olin, B., Mannervik, B. and Jones, T.A. (1993) *J.Mol.Biol., 232,* 192.
[22] Wilce, M.C.J., Board, P.G., Feil, S.C. and Parker, M.W. (1995) *EMBO J., 14,* 2133.
[23] Xiao, B., Singh, S.P., Nanduri, B., Awasthi, Y.C., Zimniak, P. and Ji, X. (1999) *Biochemistry, 38,* 11887.
[24] Shen, H., Tsuchida, S., Tamai, K. and Sato, K. (1993) *Arch.Biochem.Biophys., 300,* 137.
[25] Vega, M.C., Walsh, S.B., Mantle, T.J. and Coll, M. (1998) *J.Biol.Chem., 273,* 2844.
[26] Park, H.-J., Lee, K.-S., Cho, S.-H. and Kong, K.-H. (2001) *Bull.Korean Chem.Soc., 22,* 77.
[27] Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L. and Ketterman, A.J. (2001) *Insect Biochem.Molec.Biol., 31,* 75.
[28] Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L. and Ketterman, A.J. (2001) *Insect Biochem.Molec.Biol., 31,* 867.
[29] Udomsinprasert, R. and Ketterman, A.J. (2002) *Insect Biochem.Molec.Biol., 32,* 425.
[30] Bradford, M.M., (1976) *Anal.Biochem., 72,* 248.
[31] Prapanthadara, L., Koottathep, S., Promtet, N., Hemingway, J. and Ketterman, A.J. (1996) *Insect Biochem.Molec.Biol., 26,* 277.
[32] Berendsen, H.J.C., van der Spoel, D. and van Drunen, R. (1995) *Comp. Phys. Comm.* 91, 43.
[33] Lindahl, E., Hess, B. and van der Spoel, D. (2001) *J. Mol. Mod., 7,* 306.
[34] Guex, N. and Peitsch, M.C. (1997) *Electrophoresis, 18,* 2714.
[35] Stella, L., Nicotra, M., Ricci, G., Rosato, N. and Di Iorio, E.E. (1999) *Proteins, 37,* 1.
[36] Dragani, B., Stenberg, G., Melino, S., Petruzzelli, R., Mannervik, B. and Aceto, A. (1997) *J.Biol.Chem., 272,* 25518.
[37] Luo, J.-K., Hornby, J.A.T., Wallace, L.A., Chen, J., Armstrong, R.N. and Dirr, H.W. (2002) *Protein Science, 11,* 2208.
[38] Tasayco, M.L., Fuchs, J., Yang, X.-M., Dyalram, D. and Georgescu, R.E. (2000) *Biochemistry, 39,* 10613.
[39] Cocco, R., Stenberg, G., Dragant, B., Principe, D.R., Paludi, D., Mannervik, B. and Aceto, A. (2001) *J.Biol.Chem., 276,* 32177.

[40] Stenberg, G., Dragani, B., Cocco, R., Mannervik, B. and Aceto, A. (**2000**) *J.Biol.Chem., 275,* 10421.
[41] Gulick, A.M., Goihl, A.L. and Fahl, W.E. (**1992**) *J.Biol.Chem., 267,* 18946.
[42] Martini, F., Aceto, A., Sacchetta, P., Bucciarelli, T., Dragani, B. and Di Ilio, C. (**1993**) *Eur.J.Biochem., 218,* 845.
[43] Stevens, J.M., Hornby, J.A.T., Armstrong, R.N. and Dirr, H.W. (**1998**) *Biochemistry, 37,* 15534.
[44] Erhardt, J. and Dirr, H. (**1995**) *Eur.J.Biochem., 230,* 614.
[45] Wallace, L.A., Sluis-Cremer, N. and Dirr, H.W. (**1998**) *Biochemistry, 37,* 5320.
[46] Hornby, J.A.T., Luo, J.-K., Stevens, J.M., Wallace, L.A., Kaplan, W., Armstrong, R.N. and Dirr, H.W. (**2000**) *Biochemistry, 39,* 12336.
[47] Kong, K.-H., Inoue, H. and Takahashi, K. (**1992**) *J.Biochem., 112,* 725.
[48] Manoharan, T.H., Gulick, A.M., Puchalski, R.B., Servias, A.L. and Fahl, W.E. (**1992**) *J.Biol.Chem., 267,* 18940.
[49] Lo Bello, M., Nuccetelli, M., Chiessi, E., Lahm, A., Mazzetti, A.P., Parker, M.W., Tramontano, A., Federici, G. and Ricci, G. (**1998**) *J.Mol.Biol., 284,* 1717.

Received on April 4, 2003, accepted on May 29, 2003.

Pergamon

Insect Biochemistry and Molecular Biology 32 (2002) 425–433

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Expression and characterization of a novel class of glutathione S-transferase from *Anopheles dirus*

R. Udomsinprasert, A.J. Ketterman *

Institute of Molecular Biology and Genetics, Mahidol University, Salaya campus, Nakhon Pathom 73170, Thailand

Received 28 December 2000, received in revised form 26 June 2001, accepted 29 June 2001

Abstract

A new *Anopheles dirus* glutathione S-transferase (GST) has been obtained and named adGST4-1. Both genomic DNA and cDNA for heterologous expression were acquired. The genomic sequence was 3188 bp and consisted of the GST gene as well as flanking sequence. The flanking sequence was analyzed for possible regulatory elements that would control gene expression. In *Drosophila* several of these elements have been shown to be involved in development and cell differentiation. The deduced amino acid sequence has low identity compared with the four alternatively spliced enzymes, adGST1-1 to 1-4, from another *An. dirus* GST gene *adgstIAS1*. The percent identities are 30–40% and 11–12% comparing adGST4-1 to insect GSTs from Delta and Sigma classes, respectively. Enzyme characterization of adGST4-1 shows it to be distinct from the other *An. dirus* GSTs because of low enzyme activity for customary GST substrates including 1-chloro-2, 4-dinitrobenzene (CDNB). However, this enzyme has a greater affinity of interaction with pyrethroids compared to the other *An. dirus* GSTs. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Glutathione transferase; Anopheles dirus; Gene regulation; Promoter

1. Introduction

The glutathione S-transferases (GST) are a super-family of dimeric enzymes which currently has at least 25 possible families of GST-like proteins (Snyder and Maddison, 1997). Based on their sequences, the mammalian GSTs can be divided into seven distinct classes termed Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega (Hayes and Pulford, 1995; Board et al., 1997, 2000). The insect GSTs can be grouped into two distinct classes termed Class I or Delta class and Class II or Sigma class (Toung et al., 1990 Fournier et al., 1992). Generally amino acid sequence identity within a class is 50% or greater, while inter-class identity is less than 30% (Mannervik et al., 1992). Therefore, the GST classes span multiple species with enzymes from the same class but from different species being more similar to each other than enzymes from different classes from within a single species. Currently Delta class has only been reported in insects although Sigma class GSTs have also been reported from cephalopods (Ji et al., 1995; Board et al., 1997).

Previously we have reported *adgstIAS1* which is an *Anopheles dirus* alternatively spliced GST gene (Pongjaroenkit et al., 2001). This gene encoded four Delta class GST enzymes, adGST1-1, 1-2, 1-3, and 1-4, that we had heterologously expressed and characterized (Jirajaroenrat et al., 2001 Ketterman et al., 2001). Although splice products from the same gene, the four enzymes possessed distinct enzyme kinetic properties for substrates and inhibitors including insecticides. Several allelic variants for one of the spliced products, adGST1-1, each had single amino acid changes outside the active site that significantly affected kinetic properties of the enzymes (Ketterman et al., 2001). Molecular modeling showed that the single residue change appeared to modulate the conformations attainable by the different variants.

In this report, we describe a novel *An. dirus* GST gene including putative regulatory elements. The gene encodes a protein from a new class of insect GST that we heterologously expressed and characterized. This enzyme appears to possess little activity for customary GST substrates and may be regulated by several

* Corresponding author. Fax: +66-2-441-9906.
E-mail address: fraktu@mahidol.ac.th (A.J. Ketterman)

0965-1748/02/$ - see front matter
PII: S0965-1748(01)00119-9

elements that have been shown in *Drosophila* to be involved in development and cell differentiation.

2. Materials and methods

2.1. An. dirus genomic DNA sequencing

The recombinant bacteriophage, derived from an *An. dirus* genomic library (Pongjaroenkit et al., 2001) was partially digested with *Sal*I and *Xho*I. The DNA hybridization was performed by using the Digoxigenin (DIG)-labeled 5′ part of *adgst1-1*, the first 200 bp of the coding sequence, as the probe. The probe preparation and hybridization procedure were described previously (Pongjaroenkit et al., 2001). Positive signal DNA were subcloned into pBluescript II SK(+). The contiguous sequence of 3188 bp was obtained by automated sequencer (ABI PRISM™ 377, Perkin–Elmer) and assembled using BioEdit software.

2.2. RT-PCR and cDNA sequencing

Total RNA of fourth instar larvae of *An. dirus* was extracted by TRIzol™ Reagent. This RNA was used as the template to synthesize first strand cDNA. PCR was performed by using primers 5′CCGAGAGCATATGGATTACTACTACAGCCTC3′ and 5′CCGAGAGCATATGTCACTTTC-GGCTCGCGAC3′ and 300–500 ng of cDNA as template. Optimal PCR conditions (40 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min) were carried out in a Perkin–Elmer thermocycler to generate the coding sequence of adGST4-1. A single product of 630 bp was obtained and subcloned into pET3a (Novagen) by the *Nde*I restriction site contained in the primers, underlined above. The DNA sequencing was performed in both directions several times. The expression of the recombinant proteins was performed in *E. coli* BL21(DE3)pLysS.

2.3. Preparation of recombinant protein

The protein expression was performed as previously described (Ketterman et al., 2001). The soluble target protein was purified by HiTrap affinity columns (glutathione ligand coupled via a 10-carbon linker arm) as described in the user's instructions (Amersham Pharmacia Biotech). The bound proteins were eluted with 10 mM reduced-glutathione. The fractions containing active enzymes were concentrated using centriprep-10 ultrafiltration units (Amicon) by centrifugation at 2500*g* for 3 h, at 4°C and passed through Hitrap desalting columns (Amersham Pharmacia Biotech) to remove the glutathione. The enzymes were stored in 50 mM phosphate buffer (pH 6.5), 10 mM DTT, 40% (v/v) glycerol at −20°C. Protein was assayed by the method of Bradford using the Bio-Rad protein reagent with BSA as the standard protein (Bradford, 1976). The purity and subunit size of the enzyme preparations were confirmed by SDS-PAGE with Bio-Rad broad-range standards as molecular mass markers.

2.4. Characterization of expressed enzyme

The method for determination of GST activity with 1-chloro-2, 4-dinitrobenzene (CDNB) was described previously (Habig et al., 1974). The activity with 3 mM CDNB and 16 mM glutathione was monitored at 340 nm using a SpectraMax 250 (Molecular Devices) in 0.1 M phosphate buffer pH 6.5. This is the standard assay used for the adGST4-1 enzyme.

The kinetic studies were performed by varying the concentration of CDNB from 0.1 to 3.2 mM and glutathione from 0.25 to 20 mM. The V_{max} and K_m were determined by non-linear regression analysis using GraphPad Prism 2.01 software.

The inhibition studies were performed with the standard assay in the presence of various compounds as inhibitors. Determinations of IC_{50} were performed with ethacrynic acid and S-hexylglutathione by varying the inhibitor concentrations as previously described (Prapanthadara et al., 1996).

3. Results

3.1. Isolation and cloning of adgst4-1

Several positive signal fragments were detected after the recombinant bacteriophage was double digested with restriction enzymes, *Sal*I and *Xho*I. These fragments were subcloned into pBluescriptII SK$^+$ and sequenced. The program BLAST was used to analyze the 3188 bp contiguous sequence. The full-length gene contained two coding exons and one 59 bp intron (Fig. 1). The 794 bp down stream sequence was also analyzed for other GST coding sequences. The BLAST program was used to determine whether there were more coding exons that might yield multiple alternatively spliced products as previously observed (Pongjaroenkit et al., 2001). To determine the GST classification, the translated amino acid sequence was compared to other insect GSTs in the Genbank database by using BLAST search programs (Fig. 2). The amino acid sequence alignment was also performed to generate percent identities and similarities (Table 1). The percent identities are 33–43% and 11–12% for adGST4-1 and insect GSTs Delta class (Class I) and Sigma class (Class II), respectively. However, the percent similarities with GST Delta class were about 50–60%. Sequence alignment with other *An. dirus*, adGST1-1, 1-2, 1-3, and 1-4, showed adGST4-1 to have sequence

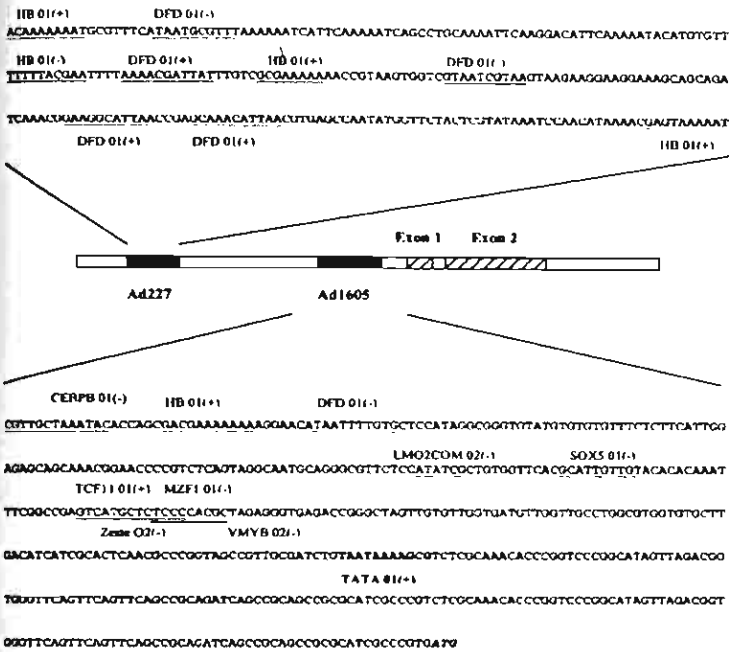


Fig. 1. A schematic of the *adgst4-1* gene isolated from an *An. dirus* genomic library. The genomic sequence for *adgst4-1* of 3188 bp is available in Genbank accession number AY014405. The two putative promoters, distal and proximal, are shown as black boxes with the protein coding exons shown as hatched boxes. Identified regulatory elements are underlined and labeled. The TATA box is shown in bold. The start site for translation is shown in bold italics.

variation even in the N-terminus which is highly conserved within a class (Fig. 2).

3.2. Promoter prediction

The 1702 bp upstream sequence of *adgst4-1* was analyzed and characterized by using a combination of two programs, TSSW (human RNA polymerase II recognition using the TRANSFAC database; http://dot.imgen.bmc.tmc.edu:9331/gene-finder/gf.html) and the MatInspector program (www.gsf.de2cgi-bin/matsearch.pl). The promoter regions were predicted by the TSSW program and the putative elements were predicted by the MatInspector program. Two putative promoters located at positions 227 and 1605 were analyzed and named Ad227 and Ad1605, respectively (Fig. 1). A TATA binding site was determined at position 1605 upstream of the coding gene. The proximal promoter Ad1605 was identified as the *adgst4-1* promoter. This putative promoter contained nine different regulatory protein binding sites (Fig. 1). These binding sites or regulatory elements may control expression of the GST gene in a tissue or stage specific manner. Additionally, the distal Ad227 promoter contained multiple Hunchback and *Dfd* recognition sites.

3.3. Expression and purification of adGST4-1

A 630 bp product from mRNA was obtained by PCR using two primers specific to the 5′ and 3′ end of the genomic sequence. The PCR product contained only coding sequence. This cDNA was subcloned into pET3a expression vector to produce a GST with a subunit size of 24,237 Da. The yield obtained after purification by glutathione ligand affinity chromatography was about 35% of the total *E. coli* lysate activity or approximately 20 mg l^{-1} of culture. During the purification of the recombinant enzyme it was observed that this GST possessed a lower affinity of interaction with the affinity column compared to other *An. dirus* GSTs in our laboratory. However, a high degree of purification, >99%, could be obtained as shown by SDS-PAGE (Fig. 3).

3.4. Characterization of adGST4-1 recombinant protein

The kinetic parameters of adGST4-1 for GSH and CDNB were determined (Table 2). Comparison of adGST4-1 with other *An. dirus* GSTs (adGST1-1, 1-2, 1-3, and 1-4) showed the adGST4-1 K_m for glutathione and CDNB was relatively high indicating low binding affinities for these substrates (Table 2). The V_m and K_m data for adGST4-1 could only be estimated because of the limitation of CDNB solubility. This also makes the k_{cat} an estimate and it is shown only for purposes of comparison. Although these kinetic parameters are estimates due to physical limitations, the values obtained were reproducible as shown in Table 2. The plots of V versus S also show the data obtained were approaching saturation thereby contributing to the reproducibility (Fig. 4). The parameters obtained, k_{cat}, k_{cat}/K_{mGSH} and k_{cat}/K_{mCDNB}, are relatively low compared to the other *An. dirus* GSTs indicating a very slow rate of turnover for CDNB and GSH. Several other substrates were used to determine activity including 1,2-dichloro-4-nitrobenzene, *p*-nitrophenethyl bromide and ethacrynic acid. However, adGST4-1 had no detectable activity for these substrates.

3.5. Inhibition study of adGST4-1

The inhibition of several compounds on CDNB conjugating activity of adGST4-1 is shown in Table 3. All compounds inhibited CDNB conjugating activity of the different *An. dirus* GSTs although to different extents. The extent of each insecticide inhibition appears to be very similar for each enzyme except the inhibition by permethrin and λ-cyhalothrin of adGST4-1 shows a significant difference from the other three adGSTs enzymes. For adGST4-1, ethacrynic acid was a good inhibitor of CDNB conjugating activity.

The IC_{50} for ethacrynic acid and S-hexylglutathione,

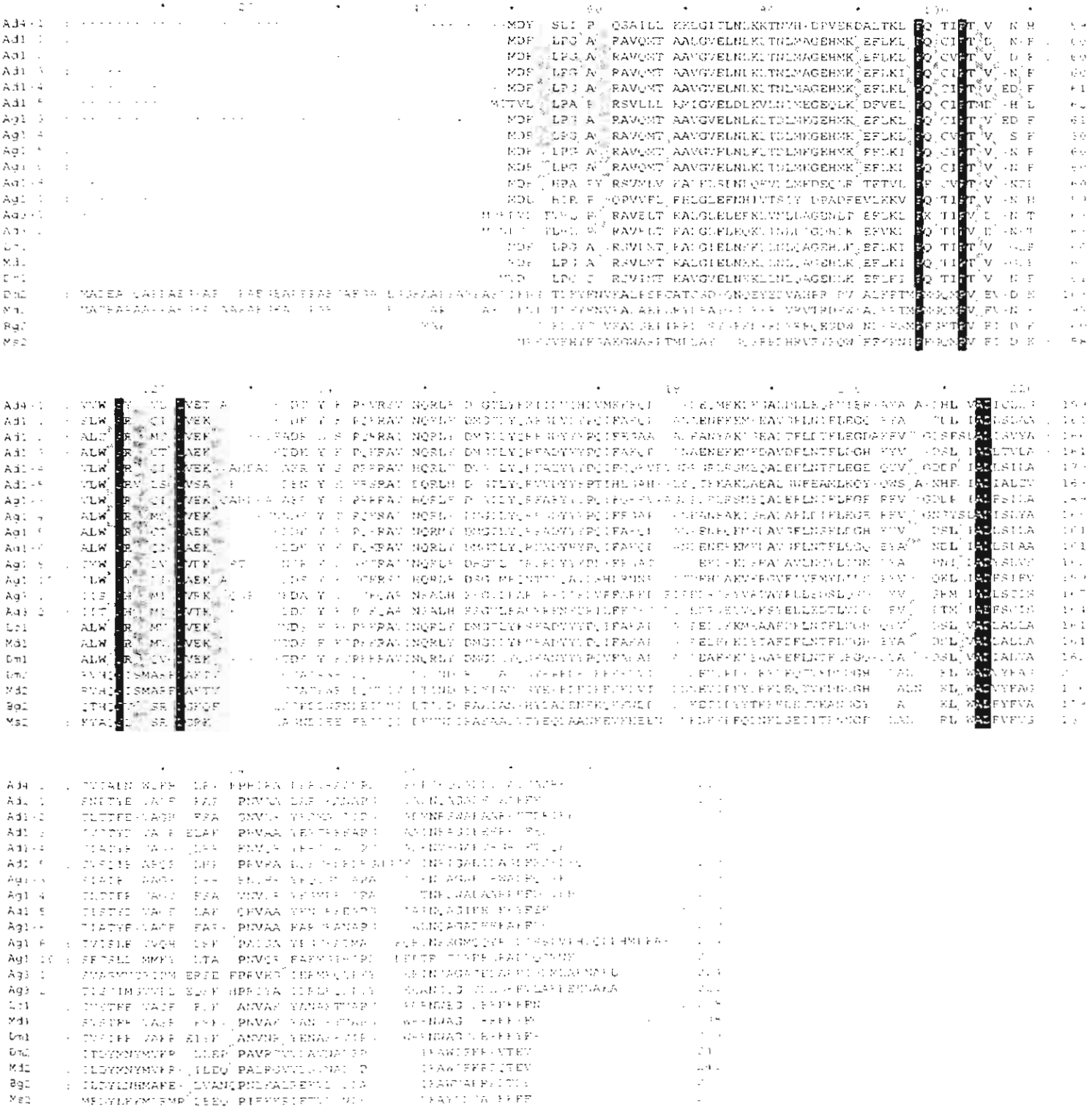


Fig. 2. The deduced amino acid sequence alignment of multiple adGSTs gene. Shading indicates degree of conservation of residue where black is 100% conserved and dark grey is >80% conserved and light grey is 60–80% conserved. The figure was produced by GeneDoc version 2.5.

23.2±3.9 μM and 126±30 μM, respectively, were determined by interpolation from the plots of fractional activity (y) versus log inhibition concentration (log [I]). This value is 100- and 13-fold greater than what was observed for adGST1-1 (Prapanthadara et al., 1996).

4. Discussion

4.1. Promotor prediction

Previously we have reported an *An. dirus* GST gene that was alternatively spliced to generate four different

Table 1
Comparison of the amino acid sequence of adGST4-1 with other insect GSTs

	Ad1-1	Ad1-2	Ad1-3	Ad1-4	Ad1-5	Ag1-3	Ag1-4	Ag1-5	Ag1-6	Ag1-8	Ag1-10	Ag3-1	Ag3-2	Lc1	Md1	Dm1	Dm2	Md2	Bg2	Ms2
	43%	33%	42%	35%	37%	33%	34%	40%	42%	37%	41%	29%	30%	38%	40%	39%	14%	15%	17%	13%
Ad4-1	60%	55%	60%	55%	58%	53%	57%	59%	61%	56%	66%	49%	52%	58%	58%	59%	27%	29%	30%	26%
		61%	72%	63%	44%	60%	62%	76%	91%	41%	38%	38%	36%	67%	68%	65%	13%	12%	16%	15%
Ad1-1		74%	87%	73%	63%	72%	74%	86%	95%	59%	54%	53%	54%	78%	78%	77%	26%	26%	29%	27%
			63%	62%	40%	60%	85%	61%	61%	40%	31%	32%	33%	58%	56%	56%	10%	9%	14%	14%
Ad1-2			78%	74%	59%	72%	91%	79%	75%	57%	53%	50%	50%	73%	72%	72%	22%	23%	29%	28%
				64%	42%	60%	62%	92%	80%	40%	37%	36%	36%	70%	70%	70%	13%	12%	16%	14%
Ad1-3				77%	62%	76%	78%	97%	90%	62%	55%	55%	56%	82%	81%	81%	25%	26%	30%	26%
					39%	84%	68%	63%	65%	37%	33%	34%	34%	57%	56%	57%	12%	10%	15%	14%
Ad1-4					56%	90%	74%	76%	75%	55%	50%	52%	54%	73%	73%	74%	22%	23%	29%	25%
						38%	40%	41%	43%	41%	32%	32%	34%	40%	41%	41%	9%	9%	12%	12%
Ad1-5						57%	62%	63%	65%	58%	50%	52%	56%	59%	59%	59%	20%	23%	25%	24%
							61%	62%	62%	36%	35%	33%	33%	54%	52%	54%	11%	10%	16%	14%
Ag1-3							74%	76%	78%	55%	51%	51%	53%	72%	71%	72%	21%	22%	29%	24%
								63%	65%	42%	34%	33%	34%	59%	57%	58%	11%	10%	14%	13%
Ag1-4								80%	76%	58%	56%	52%	51%	74%	73%	74%	22%	23%	28%	26%
									82%	40%	37%	34%	35%	68%	68%	68%	13%	12%	16%	14%
Ag1-5									91%	62%	53%	54%	56%	82%	81%	82%	24%	26%	29%	26%
										41%	38%	36%	36%	67%	68%	67%	13%	12%	16%	15%
Ag1-6										61%	55%	53%	53%	80%	81%	80%	25%	25%	29%	27%
											30%	31%	32%	41%	42%	41%	10%	10%	14%	14%
Ag1-8											52%	48%	52%	60%	60%	60%	23%	24%	28%	25%
												30%	30%	32%	32%	32%	12%	12%	18%	14%
Ag1-10												49%	50%	55%	55%	54%	25%	28%	32%	27%
													65%	35%	36%	35%	10%	10%	14%	14%
Ag3-1													83%	54%	55%	56%	22%	24%	30%	27%
														34%	35%	36%	9%	9%	13%	12%
Ag3-2														56%	56%	56%	23%	24%	30%	27%
															92%	82%	9%	8%	14%	13%
Lc1															96%	92%	23%	24%	29%	26%
																84%	10%	9%	15%	14%
Md1																92%	23%	25%	30%	28%
																	10%	10%	15%	13%
Dm1																	24%	26%	30%	28%
																		81%	38%	30%
Dm2																		84%	53%	44%
																			40%	32%
Md2																			58%	40%
																				60%
Bg2																				59%

The top number in each cell represents the percent amino acid sequence identities and the bottom number represents the percent similarity. The amino acid sequences were obtained from the GenBank database. The sequences are Ad4-1 (AY014406), Ad1-1 (AF273041), Ad1-2 (AF273038), Ad1-3 (AF273039), Ad1-4 (AF273040), Ad1-5 (AF251478), Ag1-3 (AAC79999.1), Ag1-4 (AAC79998), Ag1-5 (Q93112), Ag1-6 (Q93113), Ag1-8 (AF316637), Ag1-10 (AF316638), Ag3-1 (AF316635), Ag3-2 (AF316636), Lc1 (P42860), Md1 (P28338), Dm1 (P20432), Md2 (P46437), Dm2 (P41043), Bg2 (O18598), Ms2 (P46429). The abbreviations are Ad for *Anopheles dirus*, Ag for *Anopheles gambiae*, Lc for *Lucilia cuprina*, Md for *Musca domestica*, Dm for *Drosophila melanogaster*, Bg for *Blattella germanica*, Ms for *Manduca sexta*.

protein products (Pongjaroenkit et al., 2001). We have now obtained a novel GST gene that codes for a single expressed protein product which we name adGST4-1. The 3188 bp genomic sequence contained two coding exons and 1702 bp 5′ flanking sequence that was analyzed for promoters and regulatory elements which may control expression of this gene. Response elements are short conserved sequences that regulate expression of a gene. Several of these elements were identified in two possible promoters (Fig. 1). Most putative elements contained in the Ad227 and Ad1605 promoters have been shown in other species to be functionally involved in developmental stage regulation as well as responding to xenobiotic modulation.

Although, GSTs are involved in protecting an organism from toxic and mutagenic xenobiotics, it has been reported that the over-expression of the Pi class GST has been associated with tumor development and carcinogenesis (Batist et al., 1986). Therefore, understanding the transcriptional regulatory mechanism of these genes is of interest. Several regulating elements of GSTs have been identified. In mammals, the GSTP1 promoter included a putative AP-1 response element as well as a negative regulatory element in a multidrug resistant derivative of a human mammary carcinoma cell line (Moffat et al., 1994). Sp1 binding sites, the GC box motif, have also been shown to play a role in regulating basal levels of GSTP1 transcription (Moffat et al., 1996).

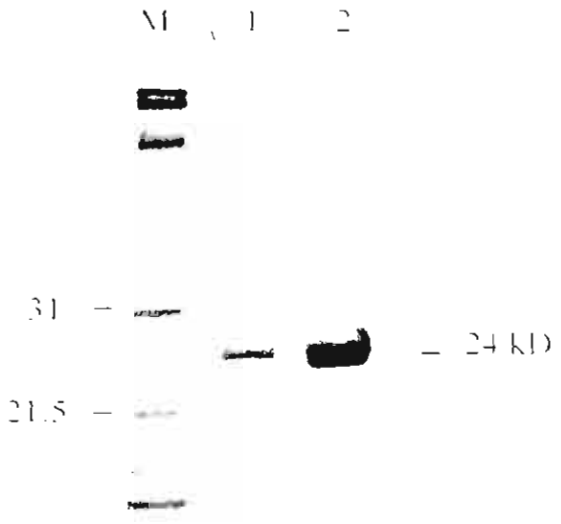


Fig. 3. Purification of recombinant protein adGST4-1. Lane M is molecular weight markers as shown in kD. Lane 1 is 3 μg and Lane 2 is 6 μg of the purified recombinant adGST4-1.

The rat GST-P gene is regulated by two enhancers and a silencer (Sakai et al., 1988). The protein bound to the silencer sequence belongs to the CCAATT/enhancer-binding protein (C/EBP) family (Osada et al., 1995). A rat Alpha class GST has been shown to be negatively regulated by C/EBP protein interaction with an antioxidant/electrophile response element (ARE/EpRE) in vascular smooth muscle cells to function as oxidative stress protection for blood vessels (Chen and Ramos, 2000).

TCF11 is a widely expressed transcription factor that binds to a subclass of AP-1 sites. The complexes of TCF11/LCR-F1/Nrf1 form heterodimers with a small Maf protein to increase stringency of specific binding to the NF-E2 site, the antioxidant response element and the heme-responsive element, and contribute to negative regulation of this specific target site (Johnsen et al., 1998). In human Pi-class GST, disruption of a putative AP-1 response element (Xia et al., 1991) within the *GSTP1* promoter abrogated *GSTP1* transcription while increased levels of GSTP1 transcription can play a major role in regulating overexpression of GSTP1-1 in multidrug-resistant cell line (Moffat et al., 1994).

Many of the putative elements contained in both *adgst4-1* promoters are DNA-binding regions for transcription factors expressed during a developmental or cell differentiation stage (Martinez-Arias and Lawrence, 1985; Siegfried and Perkins, 1990; Blair, 1994; Stauber et al., 2000). The data suggest that adGST4-1, a phase II detoxication enzyme, is expressed and regulated by

Table 2
Kinetic parameters of *Anopheles dirus* GSTs (the data are the mean±standard error of at least three separate experiments)

Kinetic parameters	**Ad4-1**	Ad1-1	Ad1-2	Ad1-3	Ad1-4
V_m (μmol $^{-1}$ min $^{-1}$ mg)	**2.2±0.3**	12.9±0.6	63.9±3.50	67.5±1.97	40.3±1.89
K_{mGSH} (mM)	**1.8±0.4**	0.86±0.2	1.30±0.15	0.40±0.05	0.83±0.08
K_{mCDNB} (mM)	**5.3±0.8**	0.10±0.03	0.21±0.03	0.10±0.01	0.52±0.67
k_{cat} (s $^{-1}$)	**0.9**	5.03	25.9	26.9	16.9
k_{cat}/K_{mGSH} (mM $^{-1}$ s $^{-1}$)	**0.5**	5.86	20	67	20
k_{cat}/K_{mCDNB} (mM $^{-1}$ s $^{-1}$)	**0.2**	48.4	121	269	32

Table 3
Inhibition of *Anopheles dirus* recombinant GSTs CDNB activity by various compounds (the GSH and CDNB concentrations were 16 and 3 mM, respectively. The data are the mean±standard deviation of at least three separate experiments, each of which was performed in duplicate)

Compounds	Concentration	% Inhibition			
	(mM)	**Ad4-1**	Ad1-2	Ad1-3	Ad1-4
DCNB	0.1	**29.8±0.2**	21.1±12.0	9.3±2.5	28.3±6.6
p-Nitrophenyl bromide	0.1	**22.5±2.2**	7.1±12.3	25.5±4.2	31.0±6.4
Cumene hydroperoxide	2.5	**43.6±5.7**	51.9±9.1	17.8±7.3	30.1±5.1
p-Nitrobenzyl chloride	1.0	**16.5±3.7**	29.1±10.5	46.1±2.5	49.8±6.7
Ethacrynic acid	0.001	**8.6±2.1**	74.7±3.0	30.9±4.5	34.5±5.3
	0.01	**39.5±5.0**	97.9±0.4	79.2±2.3	77.6±3.0
	0.1	**88.5±1.5**	100±0.0	100±0.0	100±0.0
Permethrin	0.01	**11.8±3.4**	47.4±8.5	17.4±4.7	66.3±14.4
	0.1	**28.0±3.6**	89.0±0.8	100±0.0	100±0.0
λ-cyhalothrin	0.01	**15.2±5.1**	40.1±11.3	25.0±11.8	91.5±7.6
	0.1	**25.6±1.0**	86.3±3.9	100±0.0	100±0.0

these putative elements. The distal Ad227 promoter located upstream of the Ad1605 promoter may act as an enhancer/repressor to regulate the expression of adGST4-1 as has been reported for a Pi class rat GST (Sakai et al., 1988).

4.2. Protein characterization

As suggested by amino acid sequence identities and similarities compared with other insect GSTs Class I and Class II (Table 1), adGST4-1 is very distinctive. The percent similarity of 50–60% shows that adGST4-1 is more similar to insect GST Class I or Delta class. Comparison of amino acid sequence with other *An. dirus* GSTs, 1-1 to 1-4 (Fig. 2), shows adGST4-1 has a high variation in the N-terminal, contributing to the low affinity binding with the GSH ligand during purification.

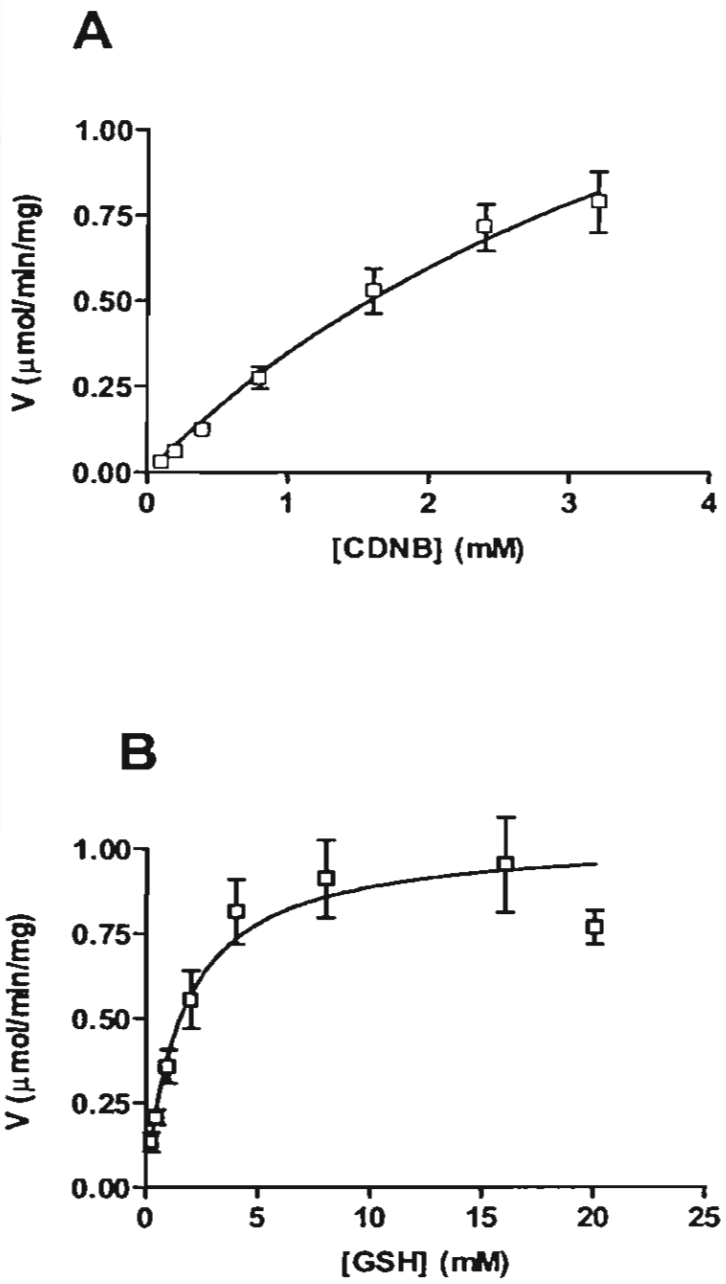


Fig. 4. Plot of velocity versus substrate concentration for kinetic constant determination of adGST4-1. (A) The glutathione concentration was fixed at 16 mM and the CDNB concentration was varied from 0.1 to 3.2 mM. (B) The CDNB concentration was fixed at 3 mM and the glutathione concentration was varied from 0.25 to 20 mM. The kinetic constants were calculated by non-linear regression. The data shown are means±SEM error bars from four independent experiments.

Studies of the enzymatic properties indicate that adGST4-1 is very different from the other known adGSTs (Table 2). The model substrate, CDNB, could be turned over only at a slow rate as described by the very low k_{cat} and k_{cat}/K_{mCDNB}. This enzyme has behavior similar to several mammalian GSTs which lack activity toward CDNB such as GST Theta class (Meyer et al., 1991), GST Zeta class (Board et al., 1997) and the new GST Omega class (Board et al., 2000). While the other *An. dirus* GSTs are more similar to mammalian GSTs in class Alpha (Schramm et al., 1984; Stenberg et al., 1991), Mu (Schramm et al., 1984; Vorachek et al., 1991) and Pi (Widersten et al., 1992). The inhibition studies (Table 3) shows that the CDNB activity of adGST4-1 can be inhibited by several compounds. Although there was no detectable enzyme activity the inhibition indicates that there is some interaction between adGST4-1 and these compounds especially for ethacrynic acid which has been shown to bind to an effector site in GST Pi class as well (Phillips and Mantle, 1993). The inhibition of CDNB conjugation by pyrethroid compounds, permethrin and λ-cyhalothrin, was also obviously different for adGST4-1 compared to other adGSTs indicating a greater affinity of interaction. The greater IC_{50} values for ethacrynic acid and S-hexylglutathione for adGST4-1 compared to the previous data for adGST1-1 (Prapanthadara et al., 1995) indicate a lower interaction with these compounds and show the inhibition characteristics of a homodimer or single affinity site for interaction (Tahir and Mannervik, 1986). Despite low CDNB conjugating activity, adGST4-1 may possess some activity for a compound that is significant in a metabolism pathway as shown by other GST classes. For example, recombinant human omega class GST (GSTO1-1) exhibited a glutathione-dependent thiol transferase activity and catalyzed glutathione-dependent reduction of dehydroascorbate (Board et al., 2000) or the novel function of human Theta GSTT2-2 with 1-menaphthylsulphate demonstrating it to be a glutathione-dependent sulphatase (Tan et al., 1996). In conclusion, the recent reports on diverse roles of GSTs in regulation of Jun N-terminal kinase (Adler et al., 1999) or in tyrosine catabolism (Dixon et al., 2000) indicate that there may be GST proteins with little traditional GST activity but having other physiological functions still to be elucidated.

Acknowledgements

This work was supported by the Thailand Research Fund.

References

Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J., Ronai, Z., 1999. Regulation of JNK signaling by GSTp. EMBO J. 18, 1321–1334.

Batist, G., Tulpule, A., Sinha, B.K., Katki, A.G., Myers, C.E., Cowan, K.H., 1986. Overexpression of a novel aniotic glutathione transferase in multidrug-resistant. J. Biol. Chem. 261, 15544–15549.

Blair, S.S., 1994. A role for the segment polarity gene shaggy-zeste white 3 in the specification of regional identity in the developing wing of Drosophila. Dev. Biol. 162, 229–244.

Board, P., Baker, R.T., Chelvanayagam, G., Jermiin, L.S., 1997. Zeta, a novel class of glutathione transferases in a range of species from plants to humans. Biochem. J. 328, 929–935.

Board, P.G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L.S., Schulte, G.K., Danley, D.E., Hoth, L.R., Griffor, M.C., Kamath, A.V., Rosner, M.H., Chrunyk, B.A., Perregaux, D.E., Gabel, C.A., Geoghegan, K.F., Pandit, J., 2000. Identification, characterization, and crystal structure of the omega class glutathione transferases. J. Biol. Chem. 275, 24798–24806.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, Y.-H., Ramos, K.S., 2000. A CCAAT/Enhancer-binding protein site within antioxidant/electrophile response element along with CREB-binding protein participate in the negative regulation of rat *GST-Ya* gene in vascular smooth muscle cells. J. Biol. Chem. 275, 27366–27376.

Dixon, D.P., Cole, D.J., Edwards, R., 2000. Characterisation of a zeta class glutathione transferase from *Arabidopsis thaliana* with a putative role in tyrosine catabolism. Arch. Biochem. Biophys. 384, 407–412.

Fournier, D., Bride, J.-M., Poirié, M., Bergé, J.-B., Plapp, F.W., 1992. Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. J. Biol. Chem. 267, 1840–1845.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hayes, J.D., Pulford, D.J., 1995. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. CRC Crit. Rev. Biochem. Mol. Biol. 30, 445–600.

Ji, X., Von Rosenvinge, E.C., Johnson, W.W., Tomarev, S.I., Paitigorsky, J., Armstrong, R.N., Gilliland, G.L., 1995. Three-dimensional structure, catalytic properties, and evolution of a sigma class glutathione transferase from squid, a progenitor of the lens S-crystallins of cephalopods. Biochemistry 34, 5317–5328.

Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L., Ketterman, A.J., 2001. Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single *Anopheles* gene. Insect Biochem. Mol. Biol. (in press).

Johnsen, O., Murphy, P., Prydz, H., Kolsto, A.-B., 1998. Interaction of the CNC-bZIP factor TCF11/LCR-F1/Nrf1 with MafG: binding-site selection and regulation of transcription. Nucleic Acids Res. 26, 512–520.

Ketterman, A.J., Prommeenate, P., Boonchauy, C., Chanama, U., Leetachewa, S., Promtet, N., Prapanthadara, L., 2001. Single amino acid changes outside the active site significantly affect activity of glutathione S-transferases. Insect Biochem. Mol. Biol. 31, 65–74.

Mannervik, B., Awasthi, Y.C., Board, P.G., Hayes, J.D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W.R., Pickett, C.B., Sato, K., Widersten, M., Wolf, C.R., 1992. Nomenclature for human glutathione transferases. Biochem. J. 282, 305–306.

Martinez-Arias, A., Lawrence, P.A., 1985. Parasegments and compartments in *Drosophila* embryo. Nature 313, 639–642.

Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G.M., Ketterer, B., 1991. Theta, a new class of glutathione transferases purified from rat and man. Biochem. J. 274, 409–414.

Moffat, G.J., McLaren, A.W., Wolf, C.R., 1994. Involvement of Jun and Fos proteins in regulating transcriptional activation of the human pi class glutathione S-transferase gene in multidrug-resistant MCF7 breast cancer cells. J. Biol. Chem. 269, 16397–16402.

Moffat, G.J., McLaren, A.W., Wolf, C.R., 1996. Sp1-mediated transcriptional activation of the human pi class glutathione S-transferase promoter. J. Biol. Chem. 271, 1054–1060.

Osada, S., Takahashi, K., Nishihira, T., Suzuki, T., Muramatsu, M., Imagawa, M., 1995. CCAAT/enhancer-binding proteins α and β interact with the silencer element in the promoter of glutathione S-transferase P gene during hepatocarcinogenesis. J. Biol. Chem. 270, 31288–31293.

Phillips, M.F., Mantle, T.J., 1993. Inactivation of mouse liver glutathione S-transferase YfYf (Pi class) by ethacrynic acid and 5,5′-dithiobis-(2-nitrobenzoic acid). Biochem. J. 294, 57–62.

Pongjaroenkit, S., Jirajaroenrat, K., Boonchauy, C., Chanama, U., Leetachewa, S., Prapanthadara, L., Ketterman, A.J., 2001. Genomic organization and putative promotors of highly conserved glutathione S-transferases originating by alternative splicing in *Anopheles dirus*. Insect Biochem. Mol. Biol. 31, 75–85.

Prapanthadara, L., Ketterman, A.J., Hemingway, J., 1995. DDT-resistance in *Anopheles gambiae* Giles from Zanzibar Tanzania based on increased DDT-dehydrochlorinase activity of glutathione S-transferases. Bull. Entomol. Res. 85, 267–274.

Prapanthadara, L., Koottathep, S., Promtet, N., Hemingway, J., Ketterman, A.J., 1996. Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (species B). Insect Biochem. Mol. Biol. 26, 277–285.

Sakai, M., Okuda, A., Muramatsu, M., 1988. Multiple regulatory elements and phorbol 12-O-tetradecanoate 13-acetate responsiveness of the rat placental glutathione transferase gene. Proc. Natl. Acad. Sci. U.S.A. 85, 9456–9460.

Schramm, V.L., McCluskey, R., Emig, F.A., Litwack, G., 1984. Kinetic studies and active site-binding properties of glutathione S-transferase using spin-labeled glutathione, a product analogue. J. Biol. Chem. 259, 714–722.

Siegfried, E., Perkins, L.A., 1990. Putative protein kinase product of the *Drosophila* segment-polarity gene *zeste-white3*. Nature 345, 825–829.

Snyder, M.J., Maddison, D.R., 1997. Molecular phylogeny of glutathione-S-transferases. DNA Cell. Biol. 16, 1373–1384.

Stauber, M., Taubert, H., Schmidt-Ott, U., 2000. Function of bicoid and hunchback homologs in the basal cyclorrhaphan fly *Megaselia* (Phoridae). Proc. Natl. Acad. Sci. U.S.A. 97, 10844–10849.

Stenberg, G., Board, P.G., Carlberg, I., Mannervik, B., 1991. Effects of directed mutagenesis on conserved arginine residues in a human class alpha glutathione transferase. Biochem. J. 274, 549–555.

Tahir, M.K., Mannervik, B., 1986. Simple inhibition studies for distinction between homodimeric and heterodimeric isoenzymes of glutathione transferase. J. Biol. Chem. 261, 1048–1051.

Tan, K.-L., Chelvanayagam, G., Parker, M.W., Board, P.G., 1996. Mutagenesis of the active site of the human theta-class glutathione transferase GSTT2-2: catalysis with different substrates involves different residues. Biochem. J. 319, 315–321.

Toung, Y.P.S., Hsieh, T.-S., Tu, C.P.D., 1990. *Drosophila* glutathione

S-transferase 1-1 shares a region of sequence homology with the maize glutathione S-transferase III. Proc. Natl. Acad. Sci. U.S.A. 87, 31–35.

Vorachek, W.R., Pearson, W.R., Rule, G.S., 1991. Cloning, expression, and characterization of a class-mu glutathione transferase from human muscle, the product of the GST4 locus. Proc. Natl. Acad. Sci. U.S.A. 88, 4443–4447.

Widersten, M., Kolm, R.H., Björnestedt, R., Mannervik, B., 1992. Contribution of five amino acid residues in the glutathione binding site to the function of human glutathione transferase P1-1. Biochem. J. 285, 377–381.

Xia, C.L., Cowell, I.G., Dixon, K.H., Pemble, S.E., Ketterer, B., Taylor, J.B., 1991. Glutathione transferase 8: its minimal, promoter and downstream cis-acting element. Biochem. Biophys. Res. Commun. 176, 233–240.

Future Plans

We will continue to perform structure function studies to increase our understanding of which amino acid residues affect enzyme specificity. We are also interested in determining the mechanism of how the elucidated amino acids contribute to the observed specificity. In addition we are also studying non-enzymatic roles of the GSTs in their interaction and regulation of the Jun N-terminal Kinase (JNK) pathway.

Publications

We have published the seven manuscripts presented in this Final Annual Report.

1. Udomsinprasert, R., Bogoyevitch, M.A. and Ketterman, A.J. (2004) Reciprocal Regulation of Glutathione *S*-Transferase Spliceforms and the *Drosophila* c-Jun N-terminal Kinase Pathway Components. *In press. Biochem. J. Immediate Publication*, DOI:10.1042/BJ20040519 *Impact Factor: 4.589*

2. Winayanuwattikun, P. and Ketterman, A.J. (2004) Catalytic and structural contributions for glutathione binding residues in a delta class glutathione S-transferase. *In press. Biochem. J. Immediate Publication*, DOI:10.1042/BJ20040697 *Impact Factor: 4.589*

3. Wongtrakul, J., Udomsinprasert, R. and Ketterman, A.J. (2003) Non-active site residues Cys69 and Asp150 affected the enzymatic properties of glutathione *S*-transferase AdGSTD3-3. *Insect Biochem. Molec. Biol.* **33**, 971-979. *Impact Factor: 2.350*

4. Vararattanavech, A. and Ketterman, A.J. (2003) Multiple roles of glutathione binding-site residues of glutathione S-transferase. *Protein and Peptide Lett.*, **10**, 441-448. *Impact Factor: 0.622*

5. Wongsantichon, J., Harnnoi, T. and Ketterman, A.J. (2003) A sensitive core region in the structure of glutathione S-transferases. *Biochem. J.* **373**, 759–765. *Impact Factor: 4.589*

6. Wongtrakul, J., Sramala, I. and Ketterman, A.J. (2003) A non-active site residue cysteine 69, of glutathione S-transferase AdGSTD3-3 has a role in stability and catalytic function. *Protein and Peptide Lett.*, **10**, 375-385. *Impact Factor: 0.622*

7. Udomsinprasert, R., and Ketterman, A.J. (2002) Expression and Characterization of a Novel Class of Glutathione S-Transferase from *Anopheles dirus*. *Insect Biochem. Molec. Biol.* **32**: 425-433. *Impact Factor: 2.350*

In the last two years we have presented ten posters of the work described in this report.

1. Piromjitpong, J., Wongsantichon, J., Winayanuwattikun, P. and Ketterman, A.J. (2003) Characterization of the contributions of the milieu of amino acids in effecting enzyme properties of glutathione S-transferases. 29th Congress on Science and Technology of Thailand, 20-22 October 2003 Khon Kaen University. SB-97P.

2. Ketterman, A. J., Harnnoi, T., Wongsantichon, J. (2003) A sensitive residue position in an inner core region in domain 2 of glutathione S-transferases. ComBio 2003 Melbourne Australia 28 Sept – 2 Oct 2003. POS-TUE-007

3. Winayanuwattikun, P. and Ketterman, A.J. (2002) Characterization of amino acid residues in the active site of *Anopheles dirus* species b glutathione S-transferase isoform 1-3. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 12

4. Vararattanavech, A., Wongsantichon, J. and Ketterman, A.J. (2002) Characterization of residues involved in the active site of glutathione S-transferase. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 13

5. Wongtrakul, J. and Ketterman, A.J. (2002) Aspartate 150 outside the active site has effect on the specificity of glutathione S-transferase adGST1-3. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 14

6. Wongsantichon, J. and Ketterman, A.J. (2002) A sensitive region in the structure of glutathione S-transferases. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 15

7. Udomsinprasert, R. and Ketterman, A.J. (2002) Studies of diptera glutathione S-transferase interaction with the stress response Jun-N-terminal kinase. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 16

8. Ketterman A.J., Wongsantichon J., Vararattanavech A. and Winayanuwattikun P. (2002) Site-directed mutagenesis of active site residues of glutathione S-transferase. ComBio 2002 Sydney Australia 29 Sept – 3 Oct 2002. POS-WED-012

9. Wongtrakul, J. and Ketterman, A.J. (2002) Cysteine 69 outside the active site has effect on the catalytic activity of glutathione S-transferase adgst1-3. At the Protein Science Network Symposium 2002, Protein Structure and Molecular Enzymology. held 29-30 August 2002 at the Center for Protein Structure and Function, Faculty of Science, Mahidol University.

10. Vararattanavech, A., Wongsantichon, J., Winayanuwattikun, P. and Ketterman, A.J. (2002) Characterization of residues involved in the active site of glutathione S-transferase. At the Protein Science Network Symposium 2002, Protein Structure and Molecular Enzymology, held 29-30 August 2002 at the Center for Protein Structure and Function, Faculty of Science, Mahidol University.

Invited Speaker

I have been an invited speaker to speak at the Department of Biochemistry, University of Western Australia.

Collaborations Domestic and International

I have several working collaborations. One collaboration is with Dr. L. Prapanthadara and Dr. Jeerang Wongtrakul at the Research Institute for Health Sciences, Chiangmai University, Chiangmai, Thailand. Drs. Prapanthadara and Wongtrakul are involved with the enzyme characterization studies and also in supplying my laboratory with *An. dirus* mosquitoes. A second collaboration is with Dr. Saengtong Pongjareankit at Maejo University. A third collaboration is with Dr. Matthew C.J. Wilce in the Structural Biology Unit, Department of Pharmacology/Crystallography Centre, University of Western Australia. A fourth collaboration is with Dr. Aaron J. Oakley at Australian National University, Canberra Australia. Drs. Wilce and Oakley are crystallographers and are currently crystallizing and elucidating the tertiary structure of the *An. dirus* recombinant GSTs that we are studying.

Collaborations within Institute

We have formed a scientific support network consisting of the following six ajarns and their respective groups (at this time this includes 30 people): Drs. C. Angsuthanasombat, G. Katzenmeier, A. Ketterman, C. Krittanai, C. Ongvarrasopone and D. Smith.

Problems

There are no unusual problems.

Comments and Suggestions

It would be helpful to receive the next funding budget quickly.