Comparative Structural Analysis of a Novel Glutathione S-Transferase (Atu5508) from Agrobacterium tumefaciens at 2.0 Å Resolution

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ABSTRACT Glutathione S-transferases (GSTs) comprise a diverse superfamily of enzymes found in organisms from all kingdoms of life. GSTs are involved in diverse processes, notably small-molecule biosynthesis or detoxification, and are frequently also used in protein engineering studies or as biotechnology tools. Here, we report the high-resolution X-ray structure of Atu5508 from the pathogenic soil bacterium Agrobacterium tumefaciens (AtGST1). Through use of comparative sequence and structural analysis of the GST superfamily, we identified local sequence and structural signatures, which allowed us to distinguish between different GST classes. This approach enables GST classification based on structure, without requiring additional biochemical or immunological data. Consequently, analysis of the atGST1 crystal structure suggests a new GST class, distinct from previously characterized GSTs, which would make it an attractive target for further biochemical studies. Proteins 2006;65:527–537. © 2006 Wiley-Liss, Inc.

Key words: glutathione transferase; X-ray crystallography; consensus sequence; local structural motif; active site; dimer interface; specificity; JCSG; structural genomics

INTRODUCTION

Agrobacterium tumefaciens is a pathogenic soil organism that is responsible for crown gall, the plant disease that causes large tumor-like growth in over 90 families of plants, and results in major agronomical losses. The Atu5508 gene from Agrobacterium tumefaciens encodes a protein with a molecular weight of 25,904 Da (residues 1–236) and a calculated isoelectric point of 4.8. Atu5508 has been annotated as a hypothetical protein in NCBI sequence databases and as a putative glutathione S-transferase (GST) in the Joint Center for Structural Genomics database (http://www.jcsg.org). Here, we present the high-resolution X-ray crystal structure of Atu5508 (AtGST1) from A. tumefaciens and results in major agronomical losses. The Atu5508 gene from Agrobacterium tumefaciens encodes a protein with a molecular weight of 25,904 Da (residues 1–236) and a calculated isoelectric point of 4.8. Atu5508 has been annotated as a hypothetical protein in NCBI sequence databases and as a putative glutathione S-transferase (GST) in the Joint Center for Structural Genomics database (http://www.jcsg.org). Here, we present the high-resolution X-ray crystal structure of Atu5508 (AtGST1) from A. tumefaciens.
Materials and Methods

Protein Production and Crystallization

A putative glutathione S-transferase, Atu5508 (GI: 15162326, Swissprot: Q8UJG9) was amplified by PCR from genomic DNA from *Agrobacterium tumefaciens* using TAQ polymerase and primers corresponding to the predicted 5′- and 3′-ends. The PCR product, representing residues 1–236 of Atu5508 was cloned into plasmid pMH1, which encodes an expression tag (MGSDKIHHHHHH) at the amino terminus of the full-length protein. The cloning was accomplished using AutoDepInputTool,17 MolProbity,18 SFcheck 4.0,19 and WHAT IF 5.0.20 The protein quaternary structure was analyzed using the PQS server.21 Figure 1(B) was adapted from a PDBsum analysis and all other figures were prepared using PyMOL (DeLano Scientific). Atomic coordinates and experimental structure factors of Atu5508 (gi:15162326) have been deposited in the PDB and are accessible under the code 2fno.

Phylogenetic Tree Construction

To assess the evolutionary relationship of atGST1 (Atu5508) to other members of the GST superfamily in SCOP, we performed a phylogenetic analysis. We selected a
subset of proteins from each of the GST structural classes in this superfamily and performed a multiple-sequence alignment using the T-coffee program.23 Sequence alignments were generated using both the complete protein sequences and those of only the N-terminal domain (which is considered less divergent and can be more reliably aligned across classes). The PDB IDs of the proteins selected from each GST class are alpha: 1ev4, 1f3a, 1ml6, 1gsd, 1guk, 1gul, 1oe7; mu: 1c72, 2gtu, 6gsv, 3gtu; pi: 11gs, 4gss, 1bay, 2gsrA; sigma: 1pgt, 1iyh, 1gsq; beta: 1a0f, 2pmt, 1f2e; delta: 1jlv, 1jlw; phi: 1aw9, 1bx9, 1axd; theta: 1jr; zeta: 1eb6, 1fw1; omega: 1ee; tau: 1gwc, 1oyj; Yeast prion protein Ure2p, nitrogen regulation fragment: 1gw; GST-like domain of elongation factor 1-gamma: 1nbh; Glutaredoxin 2: 1gt70; Chloride intracellular channel 1: 1k0m; and pfGST: 1okt. We then used the ClustalW software24 to build neighbor joining trees. To estimate the reliability of the segregations in the tree, we used the bootstrap method with 1000 trials, as implemented in the ClustalW package. Graphical representations of the trees were generated using the Treeview software.25

Profile–Profile Alignments

Atu5508 (atGST1) was aligned with all other members of the GST superfamily in the SCOP structure database (version 1.65),26 using hybrid profile-profile alignments, as implemented in HMAP.27,28 HMAP uses hybrid multidimensional profile alignment that combines sequence, secondary and tertiary structure to facilitate the detection of remote homologs. GST structures that were not in the SCOP database were also aligned with atGST1 using HMAP.

Multiple-Structure Comparisons

Representative GST structures that had similar architecture and significant sequence similarity (by HMAP profile-
Fig. 1. Crystal structure of atGST1: (A) Stereo ribbon diagram of atGST1 monomer color-coded from N-terminus (blue) to C-terminus (red). Helices (H1–H12) and β-strands (β1–β4) are labeled. (B) Diagram showing the secondary structural elements in atGST1 superimposed on its primary sequence. The α-helices, 310-helix, β-strands of sheet A, β-bulges, and γ-turns are indicated. The β-hairpin is depicted as a red loop.
The crystal structure of atGST1 [Fig. 1(A)] was determined at 2.0 Å by the MAD method. Data collection, model, and refinement statistics are summarized in Table I. The final model includes two monomers in the asymmetric unit. Each monomer contains 236 residues (residue 1 is disordered in chain A and residues 1–3 are disordered in chain B), 4 SCN$^-$ ions, and 394 water molecules. Electron density also was observed for three residues of the expression/purification tag in chain A (His$^\text{2}$, His$^\text{1}$, and His$^\text{0}$). No electron density was observed for the side chains of His$^\text{2}$, Ser$^\text{32}$, Lys$^\text{51}$, Arg$^\text{67}$, Arg$^\text{118}$, Gln$^\text{127}$, Glu$^\text{160}$, Lys$^\text{213}$, Lys$^\text{233}$ in chain A, and residues Asp$^\text{3}$, Ser$^\text{32}$, Lys$^\text{51}$, Arg$^\text{118}$, Gln$^\text{127}$, Glu$^\text{160}$, Lys$^\text{213}$, Glu$^\text{216}$, Glu$^\text{217}$ in chain B.

The Matthews' coefficient ($V_m$) is 2.61 Å$^3$/Da and the estimated solvent content is 52.4%. The Ramachandran plot, produced by MolProbity, shows that 98.25%, 99.56%, and 0.44% of the residues are in favored, allowed and disallowed regions, respectively. The only residue in the disallowed regions of the Ramachandran plot corresponds to Gln$^\text{73}$ of the A and B monomers, but has unambiguous electron density. The GST monomer consists of four β-strands (β1–β4), ten α-helices (H1–H2, H4–H5, H7–12), and two 310-helices (H3, H6) [Fig. 1(A,B)]. The total β-strand, α-helical, and 310-helical content is 5.9%, 57.3%, and 2.5%, respectively. Similar to other GSTs, atGST1 is composed of two domains: a smaller N-terminal domain (residues 2–85) followed by a short linker region (residues 86–92) and a larger C-terminal domain (residues 93–236) [Fig. 1(A)]. The N-terminal domain belongs to the thioredoxin fold, while the C-terminal domain belongs to the “GST-C-terminal domain” α-helical fold. In most GSTs, the glutathione substrate binds in the so-called “G-site”, while the electrophilic cosubstrate that becomes covalently conjugated to the glutathione binds in the usually hydrophobic “H-site”. As the two binding sites are in the dimer interface, many of the features of this interface are inevitably related to the binding and catalytic properties of GSTs.

Analysis of the crystallographic packing of Atu5508 using the PQS server identified a crystallographic dimer, for which a significant portion of the interface is formed by interactions of α-helices H3–H4 and two short loop regions. This interface buries a surface area of 1900 Å$^2$ upon complex formation [Fig. 2(B)]. Comparison of this interface with other biochemically relevant GST dimers [e.g. PDB 6gsv, Fig. 2(A)] indicates that this crystallographic dimer is the biochemically relevant oligomeric form. The interaction of the so-called “key” (Phe$^\text{56}$), after rotation of this side-chain to the position seen in 6gsv [Fig. 2(A)], would result in its interaction with the patch of hydrophobic residues in the adjacent monomer (the “lock”) and further extend this dimer interface. The asymmetric unit itself contains two distinct monomers that interact through α-helices H9–H10 and two short loop regions. This second noncrystallographic interface features a much smaller buried surface area of 358 Å$^2$ per monomer and is, therefore, less likely to be biochemically relevant.

A search using the DALI server found structural similarities to members from different GST classes. The top hit was pi GST (PDB: 1pqt, Z = 20.7) with an RMSD of 2.6 Å for this structural alignment of 204 residues (Ca atoms) with 19% sequence identity. Other high scoring DALI hits include sigma GST (PDB 1pd2, Z = 18.3), phi GST (PDB 1hqe, Z = 17.6) and beta GST (PDB 1bx9, Z = 16.8). Significant structural similarities to proteins from other GST classes (tau, omega, zeta, theta) are also among the high scoring hits (Z > 9).

Although atGST1 can be reliably classified as a GST based on sequence analysis, neither the sequence nor the structure searches found a homolog with a sequence identity of >20%. It is generally accepted that GSTs with <30% sequence identity are assigned to a separate class. However, in the absence of any biochemical data, it was not clear whether atGST1 could be classified into any of the already defined GSTs classes or whether it should define a new class based on sequence comparison alone. Compared with its distant homologs, atGST1 seemed to have some features of the alpha class and the malaria parasite pGST, but lacks some of the defining features of each of these.

These observations were corroborated by phylogenetic analysis [Fig. 3(A,B)], which indicate that atGST1 forms a new branch, not grouped within any GST families (as classified by SCOP), indicating divergence from previously characterized GST classes. It should be noted, however, that the bootstrap value for the node separating atGST1 from the neighboring branches (containing GSTs from the alpha, mu, pi, and sigma families) was 635 out of 1000 trials, with even lower values elsewhere in the tree, indicating that the significance of this separation is low. The bootstrap value increased to 735 out of 1000 trials, if only the N-terminal domain of these proteins (that are usually less divergent between classes and, therefore, can be more reliably aligned) were used in the alignment.

We then analyzed representative structures of the different GST classes using profile-based methods and multiple-structure alignments. Together with the extensive literature available on GSTs, we identified local struc-
<table>
<thead>
<tr>
<th>GST class</th>
<th>Representative PDB</th>
<th>N(^{c})atalytic tyrosine(^{a})</th>
<th>Mu loop ((\beta\2-\alpha\2))</th>
<th>Hydrophobic &quot;Key&quot; (\alpha\2-\beta\3)</th>
<th>SNAIL/TRAIL motif ((\alpha\3))</th>
<th>Catalytic Asp</th>
<th>Catalytic histidine (bacteria)</th>
<th>C' extension(^{d})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi</td>
<td>1bay</td>
<td>Y7</td>
<td>—</td>
<td>—</td>
<td>(65^{\text{SNAIL}})</td>
<td>D98</td>
<td>—</td>
<td>(+) 202–209</td>
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<tr>
<td>Mu</td>
<td>3gtu</td>
<td>Y10</td>
<td>38–44</td>
<td>F60</td>
<td>(76^{\text{SNAIL}})</td>
<td>D109</td>
<td>—</td>
<td>(+) 210–224</td>
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<tr>
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<td>Y5</td>
<td>—</td>
<td>—</td>
<td>[66^{\text{GVAIM}}]</td>
<td>(E104)</td>
<td>H106</td>
<td>—</td>
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<tr>
<td>Zeta</td>
<td>1e6b</td>
<td>(S11)</td>
<td>—</td>
<td>(M57)</td>
<td>73^{\text{SPAI}}</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Omega</td>
<td>1eem</td>
<td>(C32)</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<td>Y8</td>
<td>—</td>
<td>F51</td>
<td>67^{\text{TRAIL}}</td>
<td>D100</td>
<td>—</td>
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<td>—</td>
<td>63^{\text{SMCLA}}</td>
<td>D96</td>
<td>—</td>
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<tr>
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<td>—</td>
<td>—</td>
<td>69^{\text{SMIL}}</td>
<td>D107</td>
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<tr>
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<td>1jr</td>
<td>(S11)</td>
<td>—</td>
<td>—</td>
<td>67^{\text{SSAIL}}</td>
<td>D104</td>
<td>—</td>
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<td>37–41</td>
<td>F56</td>
<td>72^{\text{SQAI}}</td>
<td>D105</td>
<td>H107</td>
<td>—</td>
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<tr>
<td>atGST1</td>
<td>2fno</td>
<td>Y12</td>
<td>—</td>
<td>F56</td>
<td>[74^{\text{MPAI}}\text{A}}</td>
<td>D107</td>
<td>—</td>
<td>(+, (\alpha) 219–236</td>
</tr>
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Motifs marked in boldface are also found in atGST1.

\(^{a}\)Residues in round brackets mark residues in a structure that differ (in identity and position) from the noted motif but, in accordance with the literature, might substitute for its catalytic function.

\(^{b}\)The hydrophobic lock residues are in \(\alpha\4\) and \(\alpha\5\) of the opposing monomer. The key motif (with a tyrosine substituting for the phenylalanine) is present in the Pi class, according to the literature, but its dimer interface is different than in the other GST classes. However, it cannot be visualized in 1bay because of missing residues. The corresponding residue in PDB id 13gs (another representative of the same family) is Y49.

\(^{c}\)The SNAIL/TRAIL motif is present in most GSTs and contributes polar functional groups to the glutathione (G) binding site, located in the dimer interface. Square brackets denote the corresponding residues in a structure, which do not match the conserved functional motif.

\(^{d}\)A “+” denotes the presence of a C-terminal extension, “\(\alpha\)” corresponds to an \(\alpha\)-helical extension.
tural signatures that define each class, most of which consist of single residues or short, but not necessarily contiguous, structural motifs (Fig. 4 and Table II). By combining these characteristics, we could differentiate the previously characterized GST classes from one another based only on the presence or absence of these motifs. These structural signatures have corresponding functional significances, such as differences in catalytic properties or selective dimer formation only between members of a specific GST class. These structural signatures have corresponding functional significances, such as differences in catalytic properties or selective dimer formation only between members of a specific GST class. These motifs, detailed in Table II and shown in Figure 4, include (i) a tyrosine (Tyr12 in atGST1) in the N-terminal domain that is a conserved catalytic residue in several GST classes, 34 (ii) the L-loop (not present in atGST1), (iii) the hydrophobic "lock-and-key" motif, (iv) the SNAIL/TRAIL motif (not present in atGST1), (v) a catalytic aspartic acid at the beginning of the C-terminal domain, (vi) a catalytic histidine (not present in atGST1) and (vii) a divergent C-terminal region, which in some classes assumes an α-helical conformation. A conserved cis-proline35 found in all representative GSTs, is similarly conserved as Pro60 in atGST1.

Although Agrobacterium is pathogenic to plants, atGST1 is not closely related to GSTs in other bacterial or plant organisms in which GSTs of the pi, sigma, phi, and beta class are found. Comparison of the class-specific motifs of the various GST classes revealed that atGST1 is different from all of these classes. For example, comparison with beta (bacterial) GSTs showed that catalytic residues conserved in this class (Cys10 and
His106 of PDB 1a0f) are absent in atGST1. Also, beta GSTs do not contain the hydrophobic lock-and-key motif that is found in atGST1.

An HMAP profile–profile alignment determined that pfGST from the malaria parasite was one of the most similar GST structures to atGST1 (Sequence identity of ~20%). The structure of pfGST (1okt) was previously shown to define a novel GST class. Comparing this structure with atGST1 revealed that key functional and structural features are not conserved between the two
proteins; pfGST contains a variation of the “SNAIL motif”, has a short “mu loop”, and a putative catalytic histidine in position 107, all of which are not present in atGST1.

Quite a few of the class-specific motifs are located in the GST dimer interface. Dimerization is essential for stability and function, and therefore, analysis of the dimer interface is crucial in understanding GST function and specificity. One such feature, generally considered important for dimerization, is the hydrophobic “lock-and-key” motif (Figs 2(A) and 4). This motif is conserved in alpha, mu, and pi GSTs and in pfGST. The “key” is an aromatic residue in one monomer and the “lock” is a cluster of hydrophobic residues from the other interacting subunit. atGST1 seems to have this conserved feature (Phe56 corresponds to the “key” residue) in the appropriate structural location, but the aromatic side-chain points away from the dimeric interface, possibly because of the absence of bound ligands (Fig. 2(A)). This observation is consistent with previous investigations of hGST-A1-1, a member of the alpha class, where dimer formation was still maintained after mutations of this aromatic residue to a serine, but its catalytic function and dimer stability were severely reduced.

As indicated previously, Gln73 from both the A and B chains lies in the disallowed region of the Ramachandran plot. Interestingly, a structural alignment of the best scoring DALI hit (1ptg) reveals that this glutamine (Gln64 in the 1ptg structure) is oriented toward the substrate cavity, within hydrogen bonding distance to the substrate. The glutamine side-chain adopts a similar orientation in our structure. Similar side-chain orientations are observed in the structures from the pi, mu, alpha, sigma, and pfGST classes, suggesting a conserved functional role of Gln73 in substrate binding.

Almost all of the structural motifs analyzed above are functional determinants of atGST1, namely they play a significant role in catalytic function and substrate specificity. In the N-terminal domain, the complement of functional motifs that enable glutathione binding and catalysis are indeed present in atGST1 (Table II), including the conserved cis-proline (Pro60), the catalytic N-terminal tyrosine (Tyr12), and the hydrophobic “key” (Phe56). The higher conservation of the N-terminal domain and the presence of these functional motifs affirm the functionality of glutathione binding in the G site. Indeed, after testing the GST function of atGST1 using a standard functional GST assay (35–141 µg of protein, 25°C, as described in Habig and Jakoby) with GSH and p-nitrobenzyl chloride, we measured a specific activity of 0.22 ± 0.03 µmol/min/mg.

Interestingly, the presence of both the N-terminal tyrosine and the lock-and-key motifs is the hallmark of the alpha, mu, pi, and pfGST classes (which are found in mammals or in mammalian parasites). The lock-and-key motif is not characteristic of previously described bacte-
rial or plant GSTs. Moreover, an arginine residue (Arg18) is present in atGST1 in the same location and orientation as the catalytic arginine that coordinates the glutathione sulfur in the alpha class.4,41 This suggests that the catalytic mechanism for nucleophilic activation of glutathione in atGST1 is similar to that of alpha GSTs. Notably, pGST has a lysine residue, which is important for catalysis,42 at the position corresponding to the catalytic arginine.36

On the other hand, the C-terminal domain of atGST1 diverges significantly from other GSTs, suggesting differences in the substrate specificity of the H site.3,4,41 In particular, the extreme C-terminal region of atGST1 (residues 214–236), which is dissimilar in sequence and conformation to the corresponding regions of other GSTs, deserves further consideration (Fig. 4, forest green, motif VII). A corresponding (but divergent) C-terminal helix (α9) in the alpha family plays a role in the catalytic cycle and confers distinctive substrate specificity.4,40,43–45 The positioning of this C-terminal helix, which is located near the H site, alters the size of the substrate-binding pocket and, therefore, influences the specificity for the electrophilic substrate. In atGST1, the C-terminal region is longer than in most GSTs and adopts a distinctive loop-helix (H12) structure, suggesting unique catalytic properties and perhaps specific substrate recognition. Indeed, atGST1 catalyzes the conjugation of GSH to p-nitrobenzyl chloride, as do many other GSTs.40 However, no GST activity was detected with the following substrates: 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, p-nitrophenyl bromide, and trans-4-phenyl-3-buten-2-1. Thus, the structural and functional characterization of atGST1 presented here should help direct further studies of the catalytic properties and electrophilic substrate specificity of this novel GST.

In conclusion, we have combined various structural motifs identified in the known GST classes that enable us to classify GSTs at the structural level, without requiring additional biochemical or immunological results. By comparing the structural similarities and differences of atGST1 to other GSTs (as detailed in Table II), we conclude that the atGST1 structure represents a novel GST class, distinct from previously defined GST classes. Surprisingly, the catalytically relevant structural motifs of atGST1 show a closer evolutionary relationship to mammalian GSTs than to previously characterized bacterial or plant GSTs. Its distinctiveness, therefore, makes atGST1 an attractive target for further biochemical studies that will aid in a better understanding of the evolution of GSTs. In particular, the substantial differences between its active site and those of plant GSTs suggest that atGST1 may be a promising target for designing specific inhibitors against A. tumefaciens. Interestingly, we found several close homologs of atGST1 in the set of environmental sequences determined recently by the environmental sequencing project carried out by the Craig Venter Institute (https://research.venterinstitute.org/moore). Therefore, it is clear that atGST1 is not a single member of this branch—this structure likely represents a larger family, which will grow as the existing sequence databases expand. Models for GST homologs can be accessed at http://www1.jscs.org/cgi-bin/models/get_model.pl?key=15162326.

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