DROSOPHILA GLUTATHIONE S-TRANSFERASE D27: FUNCTIONAL ANALYSIS OF TWO CONSECUTIVE TYROSINES NEAR THE N-TERMINUS

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SUMMARY: The *Drosophila* glutathione *S*-transferase D27 (GST D27) has been purified and characterized after direct expression of the intronless *gst*D27 gene in *E. coli.* The GST D27 has both conjugation activity against the common substrate 1-chloro-2,4-dinitrobenzene and peroxidase activity against cumene hydroperoxide. Its pH optimum is 8.5 in 0.125 M bis-tris propane buffer at 22°C. It is more thermal labile than the human GST121. The GST D27 has two tyrosines at positions 3 and 4. Both of them appear to be important but neither of them is essential for the enzyme activity. Thus, other residues may also participate in catalysis. The two tyrosines of GST D27 could also be important in binding to GSH or *S*-hexyl GSH. Results from *in vitro* biochemical analyses were confirmed by the *in vivo* activity-based CDNB growth inhibition analyses. Our results clearly indicate that the *Drosophila* GST D isozymes are different from any of the known mammalian GSTs.
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Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of multi-functional dimeric proteins essential for xenobiotic detoxifications in many organisms. The multiple isozymes are products of a gene superfamily, consisting of at least four classes of the cytosolic GSTs and the microsomal GST in mammalian systems (1-4). The mechanism of catalysis of the conjugation reactions between GSH and an electrophile such as 1-chloro-2,4-dinitrobenzene (CDNB) requires the participation of a single tyrosine near the N-terminus in the classes alpha, mu and pi isozymes (5-12). This active site tyrosine lowers the pKa of the GSH, facilitating the formation of the glutathione thiolate anion as the nucleophile (6). The spectroscopic analysis of the rat alpha GST Y_a indicated that the active site tyrosine has a lower than normal pKa and suggested that it may contribute to general base catalysis as well (11). X-ray crystallographic models of the three different classes of GSTs revealed structural features of interactions between the active site tyrosine and GSH or its derivatives (13-19). Replacement of the active site tyrosine with phenylalanine by site-directed mutagenesis in the alpha, mu, pi GSTs resulted in mutant GSTs with drastically reduced catalytic efficiency (5-12). Recently, the importance of this active site tyrosine for enzyme activity *in vivo* has been substantiated by the phenotype of *E. coli* expressing the Y8F mutant of human GST121 in the presence of CDNB (12).

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The *Drosophila* GST D isozymes are encoded by the *gst*D multigene family which has no obvious sequence homology to any of the mammalian GSTs. The amino acid sequence identity among the functional genes ranges from 53 to 75% in pairwise comparisons (20). The enzyme activities of two members of this isozyme family purified after heterologous expression in *E. coli* revealed significant differences relative to the "universal" substrate CDNB and the pesticide DDT. While the amino acid sequences of GST D1 and GST D21 have 70% identity, only GST D1 has substantial activity toward CDNB and DDT (21). Since the order of the intronless *gst*D genes in the cluster did not reveal any gradation of sequence homology (20), it is difficult to predict the activities of other GST D isozymes from the deduced sequences. Therefore, we began to analyze other members of the gene family by heterologous expression of each gene in *E. coli* and purifying the recombinant GST for biochemical characterization. From the catalytic mechanism point of view the GST D isozymes are intriguing in that several of them have two adjacent tyrosines near the N-terminus. Therefore, it is an obvious question which one of the tyrosines or any of them is important for catalysis. We have earlier reported that the *gst*D27 gene sequence has homology to the gene for the *E. coli* stringent starvation protein (ssp) (22). In this communication, we report the biochemical characterization of recombinant GST D27 and functional analyses of the two tyrosines near its N-terminus by site-directed mutagenesis.

MATERIALS AND METHODS

NADPH, reduced GSH, GSH reductase, *S*-hexyl glutathione-linked agarose, alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and GST substrates were purchased from Sigma-Aldrich Chemical Co. *S*-Hexyl GSH and *S*-hexyl GSH-linked Sepharose 6B were prepared according to published procedures (12,21). Restriction endonucleases and T4 DNA ligase were products of Boehringer Mannheim. Exonuclease-deficient T7 DNA polymerase was kindly provided by Dr. Kenneth A. Johnson of this Department. The oligonucleotides for mutagenesis, Y3F (5' GGACTTTTTCTACCATC 3'), Y4F (5' GACTTTTACTTCCATCC-TTGC 3'), Y3F/Y4F (5' TGGACTTTTTCTTCCATCCTTG) were synthesized at the Penn State Biotechnology Institute. The methods for DNA manipulations (23), GST activity assays (24), western blot analysis (23), site-directed mutagenesis (12), and DNA sequencing (25-26) were carried out as previously described. Purification of GST D27 from *E. coli* crude extracts by *S*-hexyl GSH affinity chromatography was carried out according to the previous publication (12). Enzyme kinetic data were analyzed by the KinetAsyst™ program for the Macintosh computer. The enzyme unit is defined as μmole of product formed per minute under assay conditions.

RESULTS

Heterologous expression of the *Drosophila gst*D27 gene in *E. coli* - Analysis of the DNA sequence data of λGTDm101 revealed a concensus *E. coli* ribosome binding site sequence AGGA at 9 nucleotides upstream of the initiation codon ATG. Such an arrangement should provide a reasonably efficient signal for translational initiation (27). Therefore, a *Hind*III fragment of 836 bp which contains the complete *gst*D27 gene and including 48 nucleotides in the 5' upstream region was isolated from a subclone of λGTDm101 DNA and cloned into the *Hind*III site of the *tac*-promoter-based expression vector pKK223-3 in *E. coli* strain DH5α (28). The orientation of the *gst*D27 gene relative to the *tac* promoter was determined by the

position of the asymmetric *PstI* site in the *HindIII* fragment relative to the *PstI* site in the pKK223-3 DNA. The correct orientation for expression, designated pGTDm27-KK, generated fragments of 4708 bp and 714 bp, whereas the antisense orientation, designated pGTDm27-KK' generated fragments of 5280 bp and 142 bp upon *PstI* digestions. The correct orientation was confirmed by the detection of CDNB conjugation activity in the sonicated cell extracts. Those extracts from cells containing pGTDm27-KK' were used as the negative control. One of the pGTDm27-KK DNA was then completely sequenced by a set of oligonucleotides. The results revealed an Arg residue at position 180 instead of the Ser reported earlier (20).

The DH5α containing pGTDm27-KK was cultured at 30° for 24 h in a total volume of 6.4 liters in 8 2L flasks. The cells were concentrated by centrifugation and sonicated to obtain crude extracts. After dialysis of the extracts and *S*-hexyl GSH agarose affinity chromatography, the purified GST D27 was electrophoretically homogeneous by SDS/PAGE analysis. The affinity chromatography provided a 30-fold purification over the sonicated crude extracts. The yield was 10% with a final specific activity of 2.0 U/mg at pH 6.8 in CDNB conjugation. Rabbit antiserum against GST D27 cross-reacted with GST D21 and to a much lesser extent, GST D1 on western blots (data not shown).

Characterization of recombinant GST D27 - The *gst*D27 mRNA was a minor *gst*D mRNA species at various stages of *Drosophila* development (29). Therefore, heterologous expression of the gene provided an effective means to investigate the biochemical properties of the GST D27 enzyme. The pH optimum for CDNB conjugation is at or near pH 8.5 in 0.125 M bis-tris propane buffer. The specific activity at pH 8.5 is more than 4-fold of that at pH 6.8, which is the pH for the standard assay condition. The substrate specificities of GST D27 in Table 1 showed that it is most active toward CDNB with some activity toward *p*-nitrobenzyl chloride, ethacrynic acid, and 4-nitropyridine-*N*-oxide. It also has peroxidase activity toward cumene hydroperoxide but not toward hydrogen peroxide or ethyl hydrogen peroxide. The ratio of CDNB conjugation activity to the peroxidase activity of cumene

Table 1. Substrate Specificities of Recombinant GST D27

Substrate	Specific Activity (μmole/min/mg)	
	GST D27	hGST121
1-chloro-2,4-dinitrobenzene	1.58	143.1
1,2-Dichloro-4-nitrobenzene	<0.01	0.14
p-Nitrobenzyl chloride	0.07	0.33
Ethacrynic acid	0.03	<0.01
4-Nitropyridine-*N*-oxide	0.01	0.01
1,2-Epoxy-3-(*p*-nitrophenoxy) propane	<0.01	0.07
Cumene hydroperoxide	0.22	14.9
Hydrogen peroxide	<0.01	<0.01
Ethyl hydrogen peroxide	<0.01	<0.01

hydroperoxide is 7, which is close to that for the human GST121 (ratio=9) assayed as a positive control. The K_m's are 0.32 mM for GSH and 0.59 mM for CDNB. The k_{cat}'s are 0.94 s^{-1} for GSH and 1.65 s^{-1} for CDNB. The catalytic efficiency ($mM^{-1}s^{-1}$) is 2.95 for GSH and 2.82 for CDNB. The recombinant GST D27 was not as stable as the recombinant human GST 121. It lost 50% of the CDNB conjugation activity at 35°C after a 15 min incubation at pH 8.0 in 25 mM Tris HCl (Figure 1). This level of thermal stability, however, should be more than satisfactory for the *in vivo* function because *Drosophila* was maintained well below 35° in the laboratory.

Functional analysis of two adjacent tyrosine residues near the N-terminus of GST D27 - Since there are two tyrosines at positions 3 and 4 of GST D27 protein, it is logical to ask which one or any one of them is essential for the catalytic activity in CDNB conjugation. Therefore, three mutants, Y3F, Y4F, Y3F/Y4F, were isolated in *E. coli* DH5α after site-directed mutagenesis with specific oligonucleotides (12). Each mutation was confirmed by complete DNA sequencing of the coding sequence as well as the upstream region. The enzyme activity was determined for both sonicated crude extracts and purified proteins. The results in Table 2 showed that either GST D27-Y3F or -Y4F retained up to 50% of the CDNB conjugation activity. The purified mutant proteins with a single phenylalanine substitution also retained up to one third of the wild type GST D27 activity. The double substitutions GST D27-Y3F/Y4F still retained 17% of the CDNB conjugation activity in the crude extracts. However, the purified GST D27-Y3F/Y4F protein retained only 1.2% of the CDNB conjugation activity as the wild type GST D27. This is probably because of the instability of the purified mutant proteins. All of the three mutant proteins had weaker affinity for the *S*-hexyl GSH agarose column than the GST D27. Consequently, the yields (1.1-1.3 mg pure protein per liter culture) were considerably lower for the mutant proteins than for GST D27 (2 mg/L). The mutant GSTs also retained a fraction of the GSH peroxidase activity in GST D27.

Because of the instability of the mutant GST D27's an independent measure of their activities are needed to confirm the results in Table 2. This is accomplished by

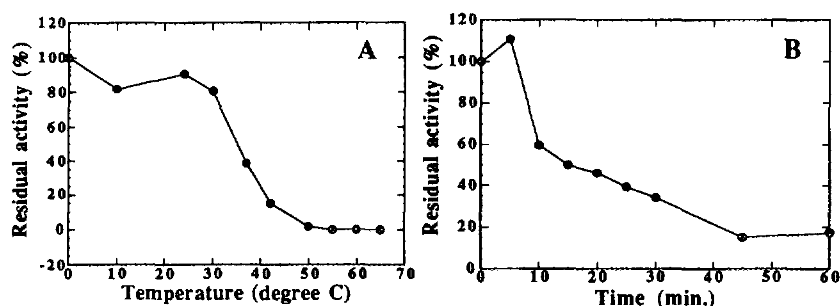

Figure 1. Thermal stability of recombinant GST D27. Panel A: GST D27 (40 μl of 0.45 mg/ml) was incubated at each temperature for 15 min. Panel B: GST D27 (200 μl of 0.45 mg/ml) was incubated at 35°C and an aliquot of 20 μl was assayed for CDNB conjugation activity at time intervals indicated.

Table 2. Comparison of Enzyme Activities between GST D27 and Its Mutants

	DH5α crude extracts (%)	Affinity Purified (%)
GST D27	100[a]	100[b]
GST D27-Y3F	56.4	20.6
GST D27-Y4F	43.7	34.0
GST D27-Y3F/Y4F	16.9	1.2

[a]The 100% value was 0.047 U/mg.
[b]The 100% value was 1.58 U/mg.

determination of plating efficiency of the various mutant strains in the presence and absence of CDNB. CDNB causes enhanced growth inhibition for *E. coli* expressing an active GST. In essence, the plating efficiency in the presence of CDNB is a measure of GST's CDNB conjugation activity *in vivo*. The higher the *in vivo* activity of a given GST the lower the plating efficiency will be in the presence of CDNB for the *E. coli* expressing it (12). The results are shown in Table 3. The ratio of plating efficiency in the presence/absence of 20 μg/ml CDNB for the antisense construct DH5α(pGTDm27-KK') is essentially unity at either 30° or 42°. In contrast, the strains carrying wild type GST D27 or any one of the three mutants have a much reduced plating efficiency. The difference in plating efficiency for each strain is from 4 to 6 orders of magnitude in favor of those without CDNB. The smaller differences in plating efficiency at 42°C than at 30°C are consistent with results on thermal stability of GST D27 in Figure 1 because it loses ~50% of the activity at 35°C in 15'. Thus, the data in Table 3 support the results of *in vitro* biochemical analysis in that the three mutants have comparable CDNB conjugation activity to GST D27, most likely within a factor of 2-5 (12).

DISCUSSION

Results from the previous section shows that the *gst*D27 gene encodes a functional GST D27, which has not only CDNB conjugation activity but also peroxidase activity against cumene hydroperoxide. The level of CDNB activities and catalytic efficiency in GST D27 is in-between those of GST D1 and GST D21 (21). The peroxidase activity against cumene

Table 3. Relative Plating Efficiency for *E. coli* DH5α Expressing GST D27 and Its Mutants in the Presence and Absence of CDNB

Plasmid	GST expressed	Plating Efficiency (+CDNB/-CDNB) 30°	42°
pGTDm27-KK'	None	1.28	0.94
pGTDm27-KK	GST D27	5.3×10^{-6}	2.0×10^{-4}
pGTDm27-Y3F	GST D27-Y3F	8.6×10^{-6}	3.6×10^{-4}
pGTDm27-Y4F	GST D27-Y4F	2.7×10^{-5}	4.0×10^{-4}
pGTDm27-Y3F/Y4F	GST D27-Y3F/Y4F	3.8×10^{-5}	3.8×10^{-4}

hydroperoxide is also comparable to those of both GST D1 and GST D21. These peroxidase activities may serve important functions when *Drosophila* is under oxidative stress, especially because the Se-dependent GSH peroxidase(s) has yet to be convincingly demonstrated in *Drosophila* (30).

The GST D27 has a pH optimum at 8.5. The pK_{a1} and pK_{a2} values are estimated from the pH profiles to be 9.64 and 7.28, respectively (data not shown). Its K_m values under the standard assay condition are comparable to those of the human GST121, GST D1 and GST D21 (12,21). The purified recombinant GST D27 is not very stable in 25 mM Tris-HCl pH 8.0 containing 1 mM GSH at 4°C, losing 30% of activity in 24 hours. It lost 20% of activity over a period 3 months at -70°C. The addition of glycerol to a final concentration of 10% helped to stabilize GST D27 at -70°C. The mutants were even less stable, losing more than 85% activity in 72 hours at 4°C.

The GST D isozymes are unique for the investigation of their mechanism of catalysis because most of them have two adjacent tyrosines near the N-terminus (20). Only the GST D25 has a single tyrosine at position 3 according to its gene sequence. This is very different from the alpha, mu, pi classes of GSTs where a single tyrosine near the N-terminus of each GST has been proven to be essential for catalysis. Their Y→F mutants lost more than 99% of the activity in the CDNB conjugation reaction (5-12). However, their ability to bind GSH or *S*-hexyl GSH remained the same because they have been purified by affinity chromatography.

Although there is no obvious sequence homology between *Drosophila* GST D27 and the alpha, mu, or pi class GSTs, several of the amino acid residues (e.g. Arg^{13}, Lys^{42}, Gln^{49}, Pro^{51}, Ser^{63}, Asp^{96}) lining up the GSH binding site in porcine lung GST pi (13) are likely to have their counterparts in GST D27 (e.g. Arg^{12}, Lys^{44}, Gln^{48}, Pro^{52}, Ser^{64}, Asp^{99}). Such conservation could indicate a similar GSH binding domain in GST D27 because the alpha, mu, and pi GSTs have similar three dimensional structures but only very limited sequence homology (18). Furthermore, the hydrophilicity plot of GST D27 (Figure 2) showed

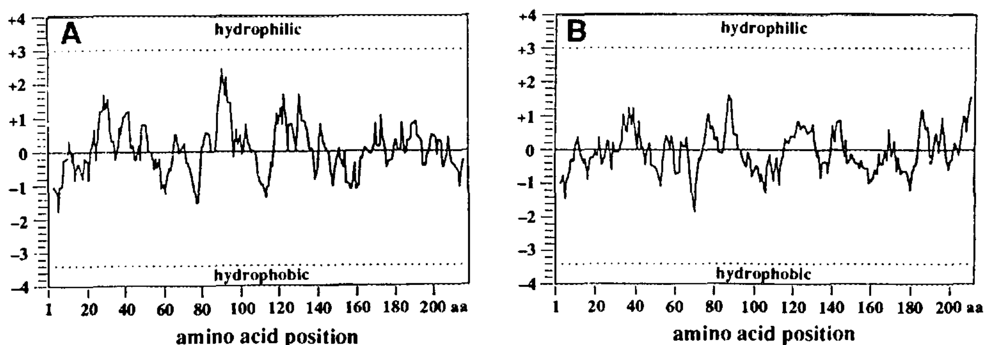

Figure 2. The hydrophilicity profiles of human GSTμ (panel A, 31) and GST D27 (panel B). Plots were constructed using the DNA Inspector IIe program from the Textco Company (West Lebanon, NH) with averaging length equal to 6. Points above the horizontal line correspond to hydrophilic regions and points below hydrophobic regions.

considerable similarity, especially in the first 100 residues, to that of the human GSTμ (31). Together with the above-mentioned biochemical properties characteristic of other GSTs in GST D27, we proceeded to investigate by site-directed mutagenesis the functions of the two adjacent tyrosines which are conserved among all but one of the GST D isozymes (20).

The Y3F, Y4F single mutants and the Y3F/Y4F double mutants of GST D27 behaved unexpectedly in that the single mutants each retained ~50% of the CDNB conjugation activity and the double mutant still retained considerable activity. The phenylalanine mutants bind less well to the *S*-hexyl GSH affinity matrix such that the yields of mutant enzymes were much reduced. These results suggest that tyrosines 3 and 4 of GST D27 contribute not only to catalysis but to GSH binding. The aromatic or hydrophobic nature of residues at these two positions may be important for GSH (*S*-hexyl GSH) binding; the mutant GST D27-Y4S did not bind to the *S*-hexyl GSH affinity column despite its 12.3% of the wild type GST D27 activity in sonicated crude extract and nearly identical plating efficiency to those in Table 3 in the presence of CDNB (2.5×10^{-5} at 30°C and 3.8×10^{-4} at 42°C). The side chains of these two adjacent tyrosines in GST D27 should be pointing at opposite orientations in the three dimensional structure. Since both of them seem to be important but neither of them appears to be absolutely essential, it is possible that the proton of the thiol in GSH may be at nearly equal distance to both -OH groups of the two tyrosines. There could be additional interaction(s) for catalysis from other residues which are yet to be identified. In summary, the GST D27 and other GST D isozymes are unique not only in their lack of sequence homology to the mammalian GSTs but also potentially in their catalytic mechanism. The *in vivo* enzyme activity based growth inhibition of *E. coli* by CDNB appears to be important and useful for characterizing these unstable mutant GSTs. Further investigation of the structure/function relationship of the GST D isozymes should be essential in understanding the physiological importance of GSTs in xenobiotic detoxification.

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