Characterization of a glutathione S-transferase and a related glutathione-binding protein from gill of the blue mussel, *Mytilus edulis*

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The major isoenzyme of glutathione S-transferase (GST 1) was purified to homogeneity from cytosolic extracts of *Mytilus edulis* gill tissue by GSH-agarose affinity chromatography followed by Mono Q ion-exchange f.p.l.c. This enzyme was particularly active with 1-chloro-2,4-dinitrobenzene, ethacrynic acid and cumene hydroperoxide as substrates. Immunoblotting and amino acid sequencing studies indicate that the enzyme belongs to the Pi class of GSTs. A related protein which binds to GSH-agarose was also purified. This GSH-binding protein did not immunoblot

with GST antisera and showed no detectable catalytic activity with GST substrates although its N-terminal sequence was similar to Mu-class GSTs. Gel-filtration chromatography indicated that GST 1 is a dimer and the GSH-binding protein a monomer. Mass spectrometry and SDS/PAGE indicate subunit molecular masses of 24 kDa (GST 1) and 25 kDa (GSH-binding protein), respectively. Both proteins have amino acid compositions typical of GSTs.

INTRODUCTION

The glutathione S-transferases (GSTs; EC 2.5.1.18) are a multifunctional family of mainly cytosolic enzymes which catalyse the conjugation of a broad range of electrophilic substrates to GSH (Chasseaud, 1979; Hayes and Wolf, 1988). The enzymes have been extensively studied in advanced mammals (reviewed by Mannervik and Danielson, 1988) but are also found in bacteria and fungi (reviewed by Sheehan and Casey, 1993a), invertebrates (reviewed by Clark, 1989), lower vertebrates (Dominey et al., 1991; Di Ilio et al., 1992; Leaver et al., 1993) and plants (Droog et al., 1993). Based on primary structures, kinetic and immunological properties, the mammalian cytosolic GSTs have been divided into Alpha, Mu, Pi (Mannervik et al., 1985) and Theta classes (Meyer et al., 1991). Since GSTs from more primitive species have not been as well-characterized as the mammalian enzymes, the exact evolutionary relationship between these classes remains unclear, although an elegant scheme has been published (Pemble and Taylor, 1992). Based on immunoblotting evidence, it has previously been reported that GSTs from a range of fungal species consist mainly of Alpha and Mu classes (Sheehan and Casey, 1993b). Evidence for Pi-class GSTs has been obtained in salmonids (Dominey et al., 1991), *Bufo bufo* embryo (Di Ilio et al., 1992) and molluscs (Tomarev et al., 1993). Such studies have extended the applicability of the classification scheme for mammalian GSTs to other species. We have recently described the separation of four proteins from the blue mussel, *Mytilus edulis*, using GSH-agarose affinity chromatography and ion-exchange f.p.l.c. (Fitzpatrick and Sheehan, 1993). In the present paper we report the biochemical characterization of the main GST from the gill of this species together with a protein which binds to GSH-agarose. This protein has N-terminal sequence similarities to GSTs but displays no catalytic activity, appears to be monomeric and does not react with GST antibodies.

MATERIALS AND METHODS

Materials

GSH-agarose was purchased from Sigma. Antisera against GSTs were from Biotrin Ltd., Dublin, Ireland.

Mussels

M. edulis were collected near Douglas estuary, Cork Harbour, Ireland. Gill tissue was carefully dissected and immediately frozen in liquid nitrogen. Tissue was stored at -70°C until required.

GST purification

Tissue (4 g) was homogenized in 4 vol. homogenization buffer (10 mM sodium phosphate buffer containing 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA and 1 mM dithiothreitol, pH 7.2) and centrifuged at 23000 *g* for 1 hr at 4°C . GSTs were affinity-purified from the resulting supernatant by GSH-agarose affinity chromatography and separated by Mono Q f.p.l.c. as described previously (Fitzpatrick and Sheehan, 1993). GST 1 was the first protein peak to elute from Mono Q and represented the bulk ($> 80\%$) of affinity extract GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The second peak to elute from Mono Q contained 60%–70% of the total protein of the affinity extract. In the present work this peak will be referred to as the 'GSH-binding protein'. These two peaks were collected and stored at -20°C until required.

Enzyme assay

GST activities with CDBN, 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy) propane

Abbreviations used: GST, glutathione S-transferase; BSP, bromosulphophthalein; TFA, trifluoroacetic acid; DI, difference index; CDBN, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; ESMS, electrospray mass spectrometry; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry.

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and *trans*-4-phenyl-3-buten-2-one as substrates were measured as described by Habig and Jakoby (1981). GST activity with acrolein as substrate was assayed as described by Berhane and Mannervik (1990). GSH peroxidase was measured with H_2O_2 and cumene hydroperoxide as substrates (Lawrence and Burk, 1976). Glyoxylase activity was assayed by the method of Jellum and Elgjo (1970). GSH reductase was determined by the procedure of Ramos-Martinez et al. (1983). Protein concentration was determined by the method of Lowry et al. (1951) using BSA as a standard.

Kinetic studies

Initial velocities with CDNB as the electrophilic substrate were determined by measuring the appearance of product, *s*-(2,4-dinitrophenyl)glutathione, at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) using a Uvikon 940 spectrophotometer. The reaction was carried out in 0.1 M sodium phosphate buffer, pH 6.5, at 25 °C. The concentration of ethanol was kept constant at 5% (v/v). The reaction was initiated by the addition of enzyme to a concentration of 1 $\mu\text{g}/\text{ml}$. The concentration of GSH was varied in the range 0.20 mM–1.50 mM and the concentration of CDNB varied in the range 0.30 mM–2.00 mM [the concentration of CDNB could not be increased above 2 mM for reasons of solubility (Phillips and Mantle, 1991)]. Non-linear regression analysis was performed on the initial rate data using the *Hyper* program run on an IBM PC. This program fits the data to a right handed hyperbola. The K_m for both substrates was then determined from replots of the parameters calculated using the *Hyper* program. IC_{50} values for inhibitors of GST 1 were determined in standard duplicate assays containing 1 mM GSH and 1 mM CDNB with 6–8 different concentrations of each inhibitor.

SDS/PAGE

SDS/PAGE was performed as described by Laemmli (1970). SDS concentration was 0.1%, and the stacker and resolving gels were 4.5% and 15% acrylamide, respectively.

Immunoblotting analysis

Polypeptides were separated on SDS/PAGE and electrophoretically transferred to nitrocellulose membranes (Atto Horizblot system) according to the method of Burnette (1981). Non-specific binding was blocked by placing the nitrocellulose membranes in 10 mM Tris/HCl/150 mM NaCl buffer, pH 8.0 (buffer A), containing 0.05% (v/v) Tween-20 (buffer B), supplemented with 1% (w/v) BSA. The membranes were then incubated with GST antisera diluted 1:200 with buffer B followed by horseradish peroxidase-conjugated goat anti-(rabbit IgG) (Promega), diluted 1:2500 with buffer B, each for 1 h with gentle agitation. After treatment with peroxidase-conjugated antibodies, the nitrocellulose membranes were washed briefly, damp-dried on filter-paper and immersed in development solution [2 ml of methanol, 8 ml of buffer A containing 4-chloro-1-naphthol (Promega) and 35 μl of 30% w/w H_2O_2]. Staining was quenched by rinsing several times in de-ionized water. Blots were dried between filter-paper and photographed.

Reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. analysis of purified GST 1 and GSH-binding protein was carried out with a Brownlee C4 column (7 μm particle size, 300 Å pore size; 30 mm $\times$ 2.1 mm) using an Applied Biosystems Inc. (ABI) model 130A microbore h.p.l.c.

The solvents were 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and 90% acetonitrile in 0.1% (v/v) TFA (solvent B). Samples were injected at 10% buffer B and a linear gradient from 10% to 79% buffer B (v/v) developed over 23 min at a flow rate of 0.3 ml/min. The column temperature was 45 °C and absorbance was monitored at 214 nm.

N-Terminal amino acid sequencing

Samples for N-terminal amino acid sequencing were dissolved in 0.1% (v/v) TFA and applied to a methanol-wetted Immobilon membrane. Analysis was carried out on either a pulsed-liquid Knauer 810 protein sequencer equipped with an ABI 140A microbore h.p.l.c. system or a Knauer 910 instrument equipped with a Knauer 64 h.p.l.c. system. The initial yield in all cases was approximately 25 pmoles.

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS)

Freeze-dried samples (5–20 pmol) were dissolved in 40% acetonitrile in 0.1% (v/v) TFA. The matrix used was alpha-cyano-4-hydroxy-sinapic acid dissolved in 70% (v/v) acetonitrile (15 $\mu\text{g}/\mu\text{l}$). Sample and matrix were mixed in a 1:1 ratio and applied to a probe tip. The sample was then inserted into a prototype laser desorption mass spectrometer for analysis (Vorm et al., 1991).

Electrospray mass spectrometry (ESMS)

Freeze-dried samples were dissolved in water:methanol:acetic acid (49:49:2, by volume) to a concentration of 0.1 mg/ml–0.5 mg/ml and applied to a single quadrupole Vestec Corp. mass spectrometer (Houston, TX, USA) at a flow rate of 1 ml/min.

Amino acid analysis

Freeze-dried samples (0.25 μg –2.5 μg) were hydrolysed at 110 °C for 20 h under vacuum using 6 M HCl and 0.1% (v/v) phenol. The hydrolysed samples were redissolved in 0.025% (w/v) EDTA and applied to an automated ABI amino acid analyser. This consisted of an ABI 420 precolumn phenylisothiocyanate derivatization unit, an ABI 130A microbore h.p.l.c. for separation of the derivatives and an ABI 920A data analysis module.

Native size determination

Samples eluted from Mono Q were passed through a Superose-12 column (Pharmacia LKB, Uppsala, Sweden). Elution volumes were determined using 50 mM sodium phosphate/150 mM NaCl, pH 7.0, as elution buffer at a flow rate of 0.5 ml/min, and compared with standard proteins of known size.

RESULTS

Immunological properties

Figure 1 shows an immunoblot of the purified *M. edulis* proteins with GST P antisera from rat. A positive reaction was observed for both *M. edulis* GST 1 and mouse GST Yp (a Pi-class enzyme) but not for rat liver affinity-purified extract which normally lacks Pi-class GST. Immunoblotting experiments with anti-Mu and anti-Alpha antisera gave no reaction with the *M. edulis* proteins but did react with the rat liver affinity-purified extract (results not shown). This indicates that mussel GST 1 belongs to the Pi class, by this criterion. The GSH-binding protein did not react with rat Pi-, Mu- or Alpha-class antisera indicating that it is likely

Figure 1 SDS/PAGE and Western-blot analysis of purified *M. edulis* proteins

(a) SDS/PAGE of GSH-agarose affinity-purified proteins. Lane 1, standard proteins with molecular masses (from top to bottom) of 67 kDa (BSA), 43 kDa (ovalbumin), 29 kDa (carbonic anhydrase), 24 kDa (trypsinogen), 20.1 kDa (soya bean trypsin inhibitor) and 14.4 kDa (α -lactalbumin); lane 2, affinity purified extract from *M. edulis* gill tissue; lane 3, Mouse Yp; lane 4, *M. edulis* GST 1; lane 5, GSH-binding protein; lane 6, affinity-purified GSTs from rat liver. (b) Western-blot of (a) with antiserum raised in rabbits against rat GST P.

that this protein is significantly different in structure to these enzymes.

N-terminal sequence analysis

The N-terminal sequences of GST1 and GSH-binding protein were determined up to residues 20 and 23, respectively, as shown in Table 1. An alignment of these sequences with those of other GSTs is shown in Table 1 and indicates that isoenzyme 1 shares 35%, 55% and 60% similarity with Pi-class enzymes from salmonid, *Bufo bufo* and mouse. A 50% similarity was observed on comparison with both octopus S-crystallin and squid GST. Comparison with the Alpha and Mu classes also shows significant similarity, 50% and 35%, respectively. The Theta class has quite a different sequence to the other three classes in the N-terminal region (Pemble and Taylor, 1992, see also Table 1). Based on this comparison, *M. edulis* GST 1 is most closely related to Pi-class GSTs.

The same comparison with the GSH-binding protein sequence reveals greatest similarity to the Mu (61%) rather than the Alpha (35%) or Pi (30%) classes. There is also considerable similarity (40%) between this protein and *M. edulis* GST 1 over the first 20 residues. A comparison of GSH-binding protein with squid and octopus S-crystallin over the first 23 residues reveals a similarity of 30% and 40%, respectively. These sequence data, together with the fact that this protein binds to GSH-agarose,

Table 1 Comparison of the N-terminal amino acid sequences of *M. edulis* gill GST 1 and GSH-binding protein with those of Alpha, Mu and Pi-class GSTs

N-terminal sequencing was carried out by Edman degradation. The sequences are aligned with those of *Bufo bufo* GST III (Di Ilio et al., 1992), salmonid GST (Dominey et al., 1991), mouse Yp (Hayes et al., 1991), squid GST (Tomarev et al., 1993) octopus S-crystallin (Tomarev et al., 1991), rat subunit 5 (Pemble and Taylor, 1992), rat subunit 3 and rat subunit 1 (Mannervik and Danielson, 1988). The consensus sequences quoted for the different GST classes are based on sequence data from the SWISS-PROT database.

Enzyme	N-Terminal sequence
<i>M. edulis</i> GST 1	PTLVYFPVRGRAEAMVRLLL
<i>Bufo bufo</i> GST III	PEYTIIFYNARGCEAMRMLLA
Salmonid GST	MPPYTITYFGVVRGAMRIMMAD
Mouse GST Yp	PPYTVVYFPVRGGCAAMRMLLA
Pi class consensus sequence	PPYTIVYFPVRGCEAMRMLLADQG
Squid GST	MPKYTLHYFPLMGRALCRFVLAAG
Octopus S-crystallin	PSYTLNYFNHRGRAEICRMLFAAG
Rat subunit 5	MVLELYDLLSQPCRAIYIFAKKN
<i>M. edulis</i> GSH-BP	PTLGWYKIRGLAQPVRLQLNYVG
Rat subunit 3	PMILGYWNVRGLTHPIRLLLEYTD
Mu class consensus sequence	PMTLGWYDIRGLAHAIIRLLLEYTD
Rat subunit 1	SGKPVLYHYFIARGMECIRWLLAAAG
Alpha class consensus sequence	AGKPVLYHYFNARGRMESIRWLLAAAG

Table 2 Amino acid compositions of *M. edulis* gill GST 1 and GSH-binding protein

Amino acid compositions were determined by amino acid analysis and are expressed as a percentage of the total. N.D. = not detectable.

Amino acid	Composition (%)	
	GST 1	GSH-binding protein
Asx	11.3	13.8
Glx	11.8	10.0
Ser	3.7	4.3
Gly	10.7	7.8
His	0.6	0.9
Arg	4.8	3.9
Thr	4.6	7.2
Ala	7.0	6.3
Pro	4.9	5.6
Tyr	4.6	4.6
Val	6.2	4.5
Met	2.5	2.2
Cys	1.1	0.4
Ile	2.0	3.1
Leu	8.9	9.4
Phe	4.7	5.8
Trp	ND	ND
Lys	8.7	8.7

would suggest some structural similarity between the GSH-binding protein and the GSTs.

Amino acid composition

The amino acid compositions of the two proteins were determined by amino acid analysis and are given in Table 2. The data indicate that GST 1 is generally similar in composition to rat Alpha- and Mu- and pig Pi-class GSTs, although showing a greater glycine and lower leucine composition than these enzymes. Comparison of amino acid compositions from two

Table 3 Comparison of specific activities of *M. edulis* GST 1 towards different substrates with mammalian GSTs from the different GST classes

The means $\pm$ SEM (minimum of three determinations) are shown for the different substrates for GST 1 from *M. edulis*. N.D. = Not detectable.

Substrate	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)					
	Alpha* Rat GST1-1	Mu* Rat GST3-3	Pi* Rat GST7-7	Pi* Human GSTP1	Theta* Rat GST5-5	GST 1 <i>M. edulis</i>
CDNB	50	58	24	105	< 0.15	10.05 $\pm$ 0.88
DCNB	< 0.04	5.3	0.048	0.11	N.D.	0.52 $\pm$ 0.05
Ethacrynic acid	0.08	0.08	3.84	0.86	N.D.	1.61 $\pm$ 0.12
1,2-Epoxy-3-(p-nitrophenoxy)propane	< 0.10	0.53	—	0.37	25.50	N.D.
<i>trans</i> -4-Phenyl-3-buten-2-one	< 0.004	0.05	0.22	0.01	< 0.001	N.D.
Acrolein	—	—	—	26.30†	—	N.D.
Cumene hydroperoxide	3.10	0.35	0.048	0.03	12.50	0.60 $\pm$ 0.08
Hydrogen peroxide	< 0.01	< 0.01	< 0.01	< 0.01	—	N.D.

* Data Compiled from Mannervik and Danielson (1988).

† Data from Berhane and Mannervik (1990).

Table 4 I_{50} values for *M. edulis* GST 1

The values shown are the means of two independent experiments each of which was performed in duplicate.

Inhibitor	I_{50} (μM)
Bromosulphthalein	4.6
Hematin	1.0
Ethacrynic acid	11.8

unrelated proteins gives a difference index (DI) value of 100, while identical proteins give a DI value of 0 (Metzger et al., 1968). GST 1 gave DI values in the range 12.60 (compared with pig Pi-class GST, Dirr et al., 1991) to 13.85 (compared with rat GST 4-4, Alin et al., 1986). The GSH-binding protein has greater percentages of glycine and tyrosine and lower percentages of arginine and leucine when compared with these enzymes. This protein gave DI values in the range 11.80 (compared with rat GST 4-4) to 14.25 (compared with Pig Pi-class GST). Interestingly, when compared with each other, the two *M. edulis* proteins analysed in this report gave a DI value of 9.10.

Substrate specificity

The specific activities of *M. edulis* GST 1 with a range of GST substrates are summarized in Table 3. When compared with other GSTs the enzyme displays modest activity with CDNB but relatively high activity with DCNB, cumene hydroperoxide and ethacrynic acid, which are frequently used as diagnostic activities for Mu, Alpha and Pi GST classes, respectively (Mannervik and Danielson, 1988). However, no activity was detected with *trans*-4-phenyl-3-buten-2-one, which is specific for Mu-class GST, or with acrolein, which has been reported as one of the best substrates for human Pi-class GST (Berhane and Mannervik, 1990). The apparent K_m^{GSH} and K_m^{CDNB} for GST 1 are 0.5 mM and 3.7 mM, respectively. The sensitivity of GST 1 to a range of inhibitors was also assessed (Table 4). IC_{50} values have been used as an additional criterion for distinguishing GST classes (Mannervik and Danielson, 1988; Ploemen et al., 1990). Pi-class GSTs are sensitive to ethacrynic acid and hematin (I_{50} approx. 4 μM) and less sensitive to bromosulphophthalein (BSP, IC_{50} approx. 20 μM). GST 1, however, shows a distinct pattern of

Table 5 Subunit structure of *M. edulis* gIII GST 1 and GSH-binding protein

Masses were determined using Superose 12 gel filtration (Pharmacia LKB, Uppsala, Sweden), *MALDI-MS or **ESMS and SDS/PAGE. Experimental details are given in the text. Values quoted are the mean of at least three independent determinations.

Method	Protein	
	GST 1 (Da)	GSH-binding protein (Da)
Subunit (SDS/PAGE)	24 500	26 000
Subunit (mass spectrometry)	24 100*	24 960**
Native M_r (gel filtration)	38 000	16 800

sensitivity to these inhibitors, being relatively more sensitive to BSP and hematin than ethacrynic acid (Table 4). These kinetic criteria appear to be insufficient for the allocation of GST 1 to any of the GST classes therefore, emphasising the importance of combining immunological, structural and catalytic data when making such classifications.

The GSH-binding protein displays no activity with any of the substrates investigated and also lacks GSH reductase and glyoxylase activity (results not shown). It is unclear what catalytic role, if any, this protein may perform as a consequence of binding GSH.

Subunit structure of purified proteins

Identification of single, high-resolution peaks on h.p.l.c. (results not shown) indicates that each protein is composed of a single subunit type (Johnson et al., 1992). This was confirmed by m.s. analysis. The subunit masses of the two proteins were determined by both SDS/PAGE and m.s. The results are given in Table 5. The mass values are within the range reported for GSTs (Mannervik and Danielson, 1988). Gel-filtration f.p.l.c. was used to determine the native masses of these enzymes. These results indicate that GST 1 is a dimer and the GSH-binding protein a monomer.

DISCUSSION

The current classification of GSTs is based primarily on enzymes purified from mammalian sources. There is considerable interest

in the evolutionary origins of the GST classes. Based mainly on DNA and deduced amino acid sequence comparisons, it has been proposed that the Theta class was the first to appear in evolution and that this gave rise to an Alpha/Mu/Pi precursor which subsequently diverged to form the Mu class and an Alpha/Pi precursor (Pemble and Taylor, 1992). In this analysis, the last major divergence was that resulting in the Alpha and Pi classes. In contrast to the volume of amino acid sequence information making such comparisons possible, relatively little is known about the structural and catalytic properties of non-mammalian GSTs. Studies have indicated that GSTs of aquatic species such as salmonids (Dominey et al., 1991) and the toad *Bufo bufo* (Di Ilio et al., 1992) belong to the Pi class. In addition, deduced amino acid sequences of lens S-crystallins and GST cDNA from molluscs such as octopus and squid appear to be most similar to Pi-class GSTs (Tomarev et al. 1991, 1993). Based on amino acid sequence alignments, it has been suggested that these molluscan proteins may comprise a separate Sigma class (Buetler and Eaton, 1992). We have previously reported that the GSH-agarose affinity-purified extract of the blue mussel, *M. edulis*, contains four main proteins separable by Mono Q ion-exchange chromatography (Fitzpatrick and Sheehan, 1993). In the present paper we report the biochemical characterization of two of these proteins which account for the bulk of the GST catalytic activity and protein, respectively, of *M. edulis* gill affinity-extracts.

GST 1 is a homodimer which seems to belong to the Pi class, based on immunological and N-terminal sequence data. Sequence alignments shown in Table 1 were constructed to highlight similarities around the catalytically-essential tyrosine residue (Kong et al., 1992). The sequence similarity to *Bufo bufo* GST III is particularly instructive with 11 residues in identical positions over the first 20 residues of GST 1. Di Ilio et al. (1992) have previously reported a microheterogeneity at residue 10 of *Bufo bufo* GST III, with valine occupying this position instead of alanine in a ratio of 1:3. In *M. edulis* GST 1, the corresponding residue is also valine, raising the sequence similarity to 60% over 20 residues. The similarity to squid GST and octopus S-crystallin is also interesting and probably indicates sequence conservation within the molluscan GSTs. It is tempting to speculate that the Sigma class identified by Buetler and Eaton (1992), which is comprised mainly of molluscan sequences, may represent a subset of the Pi class.

GST 1 is active with an interesting range of GST substrates frequently used to allocate GSTs to the main classes. However, no activity was observed with acrolein, a particularly good substrate for human GSTP1. Conversely, the enzyme was reasonably active with substrates normally thought to be diagnostic for Mu- and Alpha-class GSTs. A distinctive pattern of inhibitor sensitivities was also observed for GST 1 which is different to that of mammalian Pi-class GSTs. The immunological reactivity to rat Pi-class antisera indicates that this enzyme is more closely related to the Pi class than the Alpha or Mu classes. These results indicate that substrate specificities and inhibitor sensitivities may not be sufficient for allocation of GSTs to particular classes from sources phylogenically distant from mammals.

The second protein characterized in this study binds to GSH-agarose but displays no obvious catalytic activity with GSH. For this reason, we have called this protein the 'GSH-binding protein'. However, the protein is clearly related to GSTs (Table 1). The amino acid composition and N-terminal sequence show greatest similarities to Mu-class GSTs even though the protein does not immunoblot with antisera to the Alpha, Mu or Pi classes. Although a GST which did not immunoblot with the

main GST classes has previously been purified from *Bufo bufo* liver (Aceto et al., 1993), this enzyme showed catalytic activity with GST substrates. A 13 kDa GSH-binding protein reported as being related both to human macrophage-migration inhibitory factor and rat GST3-3 (Blocki et al., 1992) shows no significant N-terminal sequence similarity to the *M. edulis* GSH-binding protein. Also, this 13 kDa protein displays catalytic activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane, believed to be diagnostic for the Theta class, while the *M. edulis* GSH-binding protein is inactive with this substrate (Table 3). These observations indicate that these proteins are unrelated.

The precise relationship between the *M. edulis* GSH-binding protein and the GSTs remains unclear, but it is possible that it may represent a GST-like protein possessing only GSH-binding properties. A further possibility, not excluded by the present paper, is that this protein possesses GSH conjugation activity with substrate(s) other than those used in this investigation. Unfolding studies (Aceto et al., 1992; Dirr and Reinemer, 1991) indicate that a dimeric structure is essential for catalytic function. The fact that this protein is apparently a monomer may explain why no detectable catalytic activity was observed. The GSH-binding protein is presently under further investigation in our laboratory.

The results in this report indicate that, while the purified proteins are homologous to previously sequenced GSTs, they cannot readily be assigned to the present classification system. This system, which is based largely on mammalian GSTs, may not be sufficient for classification of enzymes from invertebrate sources.

The present report indicates that immunological and sequence data are apparently superior to kinetic measurements for GST classification. A more definitive answer to this problem will have to await the complete structural elucidation of further invertebrate and non-mammalian GSTs.

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