Purification and characterization of acidic glutathione S-transferase 6 from human brain

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An acidic glutathione S-transferase (GST) isoenzyme termed GST6 has been isolated from human brain, characterized and compared with other isoenzymes. The N-terminal amino acid sequence of GST6 was found to be identical with that of GST4 previously purified from human muscle. GST6 cross-reacted with antibody raised against GST4, but not with antisera raised against GST1, GST2 or GST3. The subunit M_r and pI of GST6 were found to be different from those of GST4. The present results indicate that GST6 is another member of the Mu evolutionary class which in man also includes GST1, GST4 and GST5. A minor component that co-purified with GST6 was shown to have an N-terminal sequence similar to, but not identical with, that of GST3. This isoenzyme may be an additional member of the Pi evolutionary class.

INTRODUCTION

The glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of isoenzymes that catalyse the conjugation reaction between GSH and various electrophiles, including many carcinogens and mutagens (Jakoby & Habig, 1980). In mammalian species the cytosolic GST isoenzymes can be classified into at least three evolutionary classes, termed Alpha, Mu and Pi (Mannervik *et al.*, 1985). Board (1981*a,b*) originally reported genetic relationships between several isoenzymes in human liver and erythrocytes. It was suggested that human liver contains the products of two autosomal loci, *GST1* and *GST2*, and erythrocytes contain the product of a third locus, *GST3*. The GST2 isoenzymes, also termed B₁B₁ and B₂B₂ by others (Stockman *et al.*, 1985) have subsequently been shown to be the products of two similar closely linked, but distinct, genes (Tu & Qian, 1986; Board & Pierce, 1987; Rhoads *et al.*, 1987; Hayes *et al.*, 1989). The GST1, GST2 and GST3 isoenzymes were shown to be members of the Mu, Alpha and Pi evolutionary classes respectively (Suzuki *et al.*, 1987; Board & Webb, 1987; Board & Pierce, 1987; Board *et al.*, 1989). In addition, the muscle-specific isoenzyme, GST4, and the brain-specific isoenzyme, GST5, have also been shown to be members of the Mu evolutionary class (Suzuki *et al.*, 1987; Board *et al.*, 1988).

In previous studies (Suzuki *et al.*, 1987), we identified another isoenzyme, termed GST6, that is the most anionic human GST reported so far. In order to define the interrelationships between GST6 and other human GSTs, we have isolated and characterized GST6 from human brain and compared it with other human GST isoenzymes.

MATERIALS AND METHODS

The GST activity was determined spectrophotometrically at 340 nm with 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates (Habig *et al.*, 1974). Protein concentration was measured with Coomassie Brilliant Blue G-250 (Sigma) by the method of Bradford (1976). Horizontal starch-gel electrophoresis or agarose/starch-gel electrophoresis was carried out as previously described (Board, 1980; Smith *et al.*, 1986; Suzuki *et al.*,

1987). SDS/PAGE was performed in 12.5 % gels by the method of Laemmli (1970).

Purification of GST6 from human brain

GST6 was isolated from human brain which was obtained at post mortem from an adult male who died as the result of a myocardial infarction. Human brain (663.88 g) was homogenized in 4 vol. of 10 mM-Tris/HCl buffer, pH 8.0, by means of an electric blender and a motor-driven Teflon/glass homogenizer. The homogenate was centrifuged at 10000 *g* for 2 h to remove cell debris, and the supernatant was filtered through a plug of cotton to remove lipid material. The volume of the extract was 2410 ml. Solid (NH₄)₂SO₄ was added to the extract to make 40 % saturation, and the mixture was gently stirred overnight at 4 °C. The mixture was centrifuged at 10000 *g* for 30 min, and the precipitate was collected. The precipitate was dissolved with an adequate volume of 10 mM-Tris/HCl buffer, pH 8.0, and dialysed against five changes of 5 litres of the Tris/HCl buffer. The dialysed sample (420 ml) was divided into two equal parts, and each sample was applied to a DEAE-cellulose column (3.5 cm × 30 cm) equilibrated with 10 mM-Tris/HCl, pH 8.0. After washing extensively to remove unbound protein, a 2-litre linear gradient between 0 and 0.1 M-KCl in the Tris/HCl buffer was passed through the column. A second gradient between 0.1 M- and 0.3 M-KCl was applied after the first gradient. The active fractions were pooled in five groups: peak I, fractions 24–40 (410 ml); peak II, fractions 140–157 (460 ml); peak III, fractions 161–171 (280 ml); peak IV, fractions 175–187 (280 ml); peak V, fractions 191–229 (925 ml). Each pooled sample was concentrated by ultrafiltration with an Amicon PM 10 membrane. The concentrated samples were dialysed against the Tris/HCl buffer. Aliquots were examined by starch-gel electrophoresis and specific GST staining (Board, 1980) to identify fractions containing different isoenzymes. The last active fraction, peak V, contained GST6. The GST6 fraction was dialysed against 20 mM-phosphate buffer, pH 7.0. The sample (130 ml) was applied to an Affi-Gel Blue (Bio-Rad) column (2.3 cm × 14 cm). After washing extensively with the phosphate buffer, 0.5 M-KCl in the phosphate buffer was applied to the column and the active fractions were collected; their pooled volume was 220 ml. The sample was again concentrated by

Table 1. Purification of GST6 from human brain

Stage	Total volume (ml)	Total protein (mg)	Activity (unit/ml)*	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	2410	14493.7	0.298	718.18	0.050	100
DEAE-cellulose†	925	1660.38	0.136	125.80	0.076	17.5
Affi-Gel Blue‡	220	370.04	0.146	32.12	0.087	4.5
GSH-agarose§	39	1.27	0.121	4.72	3.717	0.66

* The enzyme activity was measured at 37 °C using 0.2 mM-CDNB and 0.2 mM-GSH as substrates; a unit is 1 μ mol of conjugate/min.

† The chromatography was carried out at pH 8.0, and a KCl linear gradient was applied.

‡ The chromatography was carried out at pH 7.0 and 0.5 M-KCl was applied.

§ The affinity chromatography was performed at pH 7.0, and the active eluant was collected by application of 5 mM-GSH, pH 9.6.

ultrafiltration (Amicon membrane) and dialysed against 20 mM-phosphate buffer, pH 7.0. The dialysed sample (18 ml) was applied to a GSH-agarose (Sigma) column (2.3 cm $\times$ 6 cm) equilibrated with 20 mM-phosphate buffer, pH 7.0. The column was washed extensively with 50 mM-Tris, pH 9.6, and the GST6 was eluted subsequently with 50 mM-Tris, pH 9.6, containing 5 mM-GSH. The sample was kept in the presence of 30–40% glycerol at –20 °C until it was used.

Specific activities of GST6 were determined with several compounds which have been previously reported to be substrates for rat GSTs (Habig *et al.*, 1974). Glutathione peroxidase activity of the purified samples was measured by the method described by Beutler (1974) with H₂O₂, t-butyl hydroperoxide and cumene hydroperoxide as substrates.

The inhibitory effects of cholate, chenodeoxycholate, lithocholate 3-sulphate, ellagic acid and bromosulphophthalein on GST6 were examined in the CDNB assay system.

Immunoblotting of purified isoenzymes was performed by the methods of Towbin *et al.* (1979) and Board (1984). Antibodies raised against purified GST1, GST2, GST3 and GST4 which were used in these experiments have been reported in our previous work (Suzuki *et al.*, 1987; Board *et al.*, 1988).

The N-terminal amino-acid-sequence analysis was carried out using an Applied Biosystems model 477A sequencer. A portion of the sample was passively absorbed from the glycerol solution (2 days at 4 °C) on to Applied Biosystems ProBlot membrane (0.4 cm²). After washing in glass-distilled water, the membrane was positioned in the cartridge of the sequencer over a Polybrene-treated glass-fibre disc. After sequencing to 30 residues, the protein remaining on the disc and membrane was hydrolysed in 6 M-HCl for 22 h at 110 °C, and the total amount of protein determined by amino acid analysis using a Beckman 6300 amino acid analyser.

RESULTS

A summary of the purification is shown in Table 1. GST6 was separated from less acidic GST isoenzymes by chromatography on DEAE-cellulose (Fig. 1) and separated from other protein by chromatography on Affi-Gel Blue and GSH-agarose. GST activity was eluted from the GSH-agarose column as a single peak. The GST6 fraction represents less than 5% of the total GST activity with CDNB in this human brain sample.

The purified GST6 clearly had increased electrophoretic mobility when compared with GST4 from muscle under non-denaturing conditions in agarose gels (Fig. 2). This difference largely reflects the pI values for GST4 and GST6, which we previously reported (Suzuki *et al.*, 1987). Denaturing SDS/PAGE of purified GST6 is shown in Figs. 3 and 4. the subunit M_r of GST6 was calculated to be 27 500, which is slightly lower than

Fig. 1. DEAE-cellulose chromatography

A homogenate of human brain was made in 10 mM-Tris/HCl buffer, pH 8.0. After concentration by (NH₄)₂SO₄ precipitation and dialysis, the sample (420 ml) was divided into two equal parts and each sample was applied to a column (3.5 cm $\times$ 30 cm) of DEAE-cellulose equilibrated in 10 mM-Tris/HCl buffer, pH 8.0. The two columns were washed extensively, and a 2-litre linear gradient between 0 and 0.1 M-KCl in 10 mM-Tris/HCl buffer, and then a second gradient between 0.1 M- and 0.3 M-KCl, was applied to each column. Fractions 191–229 (925 ml) were pooled for GST6.

Fig. 2. Agarose/starch-gel electrophoresis of purified GST6 and GST4

Lanes 1 and 4, haemoglobin (Hb) marker; lanes 2 and 5, purified GST6 from brain; lane 3, purified GST4 from muscle.

that of GST1 and higher than that of GST4. Because the difference in M_r between GST6 and GST4 subunits is relatively minor, this experiment was repeated on several occasions, and the slightly increased mobility of GST4 subunits has always been observed (Fig. 4). Examination of the lanes containing GST6 in Fig. 3 shows that the preparation contains two proteins, a quantitatively minor (~10–20%), but slightly larger, component, and a smaller, but quantitatively major, band. Several experiments, including that illustrated in Fig. 4, have shown that it is the quantitatively major component that cross-reacts with the antiserum raised against denatured GST4. It is this major component that is referred to as GST6. Protein

Fig. 3. SDS/PAGE of purified GST6

Lanes 1 and 5, GST6 preparation (the minor form described in the text is indicated by an arrow); lane 2, partially purified GST4 containing GST3 (the GST3 band is very weak and is indicated by an arrow); lane 3, GST1; lane 4, M_r markers.

Fig. 4. Immunoblotting of GST6 and GST4 with anti-GST4 serum

Lanes 1, 2, 4, 6 and 7, GST4; lanes 3 and 5, GST6.

	5	10	15	20	25	30
GST6	PMTLGYWNI	RGLAHSIR	LLLEYTD	SSYEKK		
GST4 ^a	PMTLGYWNI	RGLAHSIR	LLLEYTD	*****		
GST1 ^{a,b}	PMILGYWDI	RGLAHSIR	LLLEYTD	SSYEKK		
GST3 ^{a,c}	PPYTVVYFP	VRGRCAAL	RMLLADQ	QGSWKEE		
Minor form	APYTVVYFP	VRG*****				
FAEE synthase ^d	APYTVVYFP	VRGRXKAL	RMLXAD	*****		
GST2 ^e	AEKPKLHYF	NARGRMEST	RWLLAAAG	VEFEKK		

Fig. 5. Comparison of the N-terminal amino acid sequences of GST6, the minor form and other GSTs

References: ^a, the sequence was derived from that given by Board *et al.* (1988); ^b, ^c and ^e, the sequences were deduced from cDNA sequences reported by Seidegård *et al.* (1988), Kano *et al.* (1987) and Board & Webb (1987) respectively; ^d, the sequence was derived from that given by Bora *et al.* (1989). *, not determined.

sequencing of the major component (GST6; ~ 300 pmol) was taken to 30 residues with a repetitive yield of 94–95%, and from the amino acid analysis after the run, the absolute yield was ~ 80%. Details of the background corrected data for that amino sequence are (amino acids in one-letter code): residue 1, P,

Table 2. IC_{50} values of GST6 preparation and other human GSTs with several inhibitors

Inhibitor	Isoenzyme...	IC_{50} *			
		GST3	GST1	GST4	GST6
Cholate		> 200	100	> 200	> 200
Chenodeoxycholate		> 200	50	> 200	50
Lithocholate 3-sulphate		30	20	20	20
Ellagic acid		105	90	50	8
Bromosulphophthalein		50	6	8	4

* The IC_{50} value is taken as the concentration (μM) of the inhibitor producing 50% inhibition of enzyme activity.

225 pmol; 2, M, 336; 3, T, 283; 4, L, 257; 5, G, 224; 6, Y, 170; 7, W, 53; 8, N, 98; 9, I, 223; 10, R, 14; 11, G, 168; 12, L, 196; 13, A, 209; 14, H, 24; 15, S, 65; 16, I, 135; 17, R, 16; 18, L, 106; 19, L, 132; 20, L, 165; 21, E, 37; 22, Y, 70; 23, T, 104; 24, D, 28; 25, S, 30; 26, S, 48; 27, Y, 31; 28, E, 12; 29, K, 9; 30, K, 25. The data for the minor component are: residue 1, A, 44.4 pmol; 2, P, 80* (*includes carry-over from major sequence); 3, Y, 21; 4, T, 55*; 5, V, 32; 6, V, 35; 7, Y, 40*; 8, F, 26; 9, P, 20; 10, V, 22; 11, R13*, 12, G, 50*. The GST6 sequence (major component) is compared with those of GSTs 1–4 in Fig. 5 and was found to be identical with the first 24 amino acid residues of GST4.

The sequence of the minor component was also determined, although with lower reliability, because of its relatively low level and interference by carry-over/lag from the major sequence. This sequence is quite different from that of GST6, and resembles that of GST3. Notably, the first residue is alanine, compared with proline in the GST3 sequence. Because this minor component clearly has a sequence resembling a GST, it may represent a distinct, and as yet uncharacterized, GST isoenzyme.

Although it is a mixture of two separate isoenzymes, the activity of GST6 preparation with CDNB was relatively low (3.7 $\mu mol/min$ per mg), and these isoenzymes did not have broad substrate specificity. Weak activity was detected with 1,2-dichloro-4-nitrobenzene (0.41 $\mu mol/min$ per mg), but no activity could be detected with 1,2-epoxy-3-(*p*-nitrophenoxy)propane, ethacrynic acid, *trans*-4-phenyl-3-buten-2-one or bromosulphophthalein. This mixed preparation did not exhibit any glutathione peroxidase activity when cumene hydroperoxide, *t*-butyl hydroperoxide and H_2O_2 were used as substrates.

The inhibitory effects of five chemicals on the GST6 preparation are shown in Table 2. Ellagic acid and bromosulphophthalein were found to be stronger inhibitors of GST6 than of GST3. With the exception of ellagic acid, the inhibition of GST6 was similar to that of GST1 and GST4. This probably reflects the preponderance of GST6 in the preparation.

Antibody raised against denatured GST4 was found to cross-react with GST6, as shown by immunoblotting in Fig. 4. Similar experiments using antiserum raised against GST1, GST2 or GST3 failed to show any cross-reactivity with GST6, the major component, or the minor component, that co-purified with GST-6.

DISCUSSION

The existence of a highly acidic GST isoenzyme termed GST6 was first reported by Suzuki *et al.* (1987). The studies here provide a more detailed characterization of GST6 than has previously been reported. There are a number of characteristics that indicate that GST6 is closely related to GST4, a muscle-

specific isoenzyme (Suzuki *et al.*, 1987; Board *et al.*, 1988). It is shown here that GST6 and GST4 have identical *N*-terminal sequences which, in turn, are very similar to those of GST1. Thus it appears that GST6 is a member of the Mu evolutionary class. Interestingly, antiserum raised against denatured GST4 isolated from SDS/polyacrylamide gels was able to recognize GST6. In our previous studies (Suzuki *et al.*, 1987) we failed to find any evidence of cross-reaction between antiserum to GST1 and GST6 or GST4. However, more recent investigations (Board *et al.*, 1988) have shown that antiserum to denatured GST4 cross-reacts with GST1, thus confirming the basic relationship between these three isoenzymes.

Considering the evidence of identical *N*-terminal sequences and immunological identity, it is possible that GST6 and GST4 are allelic products of the same gene locus. However, there is evidence that they have a separate genetic origin. The *pI* values for GST4 and GST6 are clearly different (5.2 and 4.25 respectively) (Suzuki *et al.*, 1987), and there is a minor, but reproducible, difference in their M_r . In particular, their expression in different tissues differs substantially, and argues strongly against an allelic relationship. In our previous studies, Suzuki *et al.* (1987) showed that GST6 was only detected in tissues from some individuals (8/11). In those individuals that expressed GST6, it was detected in all tissues tested, except erythrocytes, and only in trace amounts in the liver. In contrast, GST4 was expressed in all individuals studied, but only in skeletal and cardiac muscle. Furthermore, if GSTs 4 and 6 were allelic, one would expect to find heterodimers in tissues where both forms are expressed, as occurs in the case of GST1 types 1 and 2 (Board, 1981a). Thus, taken individually, these points do not prove the existence of separate gene loci for GST4 and GST6; however, when considered together, the weight of evidence suggests that an independent genetic origin is the most likely explanation. A similar situation exists within the human Alpha Class isoenzymes, where it has been shown that two isoenzymes termed GST2 type 1 and GST2 type 2 (or B₁B₁ and B₂B₂) have immunological identity, the same subunit M_r , but differ in only 11 amino acid residues, and are considered to be the product of separate genes (Tu & Qian, 1986; Board & Pierce, 1987; Rhoads *et al.*, 1987; Hayes *et al.*, 1989). Tsuchida *et al.* (1990) have identified five isoenzymes of the Mu evolutionary class in human aorta and heart; these isoenzymes, however, seem to be different from GST6 according to their *pI* and *N*-terminal amino acid sequences. These data therefore suggest that there may be four or five Mu Class genes expressed in man.

The nature of the minor component that co-purified with GST6 is of interest. This protein did not cross-react with antiserum directed against GSTs 1, 2, 3, or 4, yet showed considerable sequence identity with GST3, the human Pi Class isoenzyme. GST3 has a much smaller subunit M_r than that of the protein isolated here, and although there is considerable sequence identity, the *N*-terminal residues of these two proteins differ. It therefore seems likely that the minor form is a separate member of the Pi evolutionary class. Recently, Bora *et al.* (1989) have shown that a fatty acid ethyl ester (FAEE) synthase has some GST activity and has an *N*-terminal sequence that starts with alanine and is the same as that determined here for the minor form (Fig. 5). Further studies are needed to determine the relationships between the GST gene family and the FAEE synthases.

The physical relationship between GST6 and the minor form remains open to question. Although heterodimers formed from the products of different GST genes are known to occur in man (Board & Pierce, 1987; Hayes *et al.*, 1989) and rats (cf. Mannervik & Danielson, 1988), these heterodimers are only between members of the same class. Although GST6 and the minor form have been co-purified, the large difference in their relative abundance and their apparent membership of different classes suggests that their co-purification is due to their similarity in *pI* and affinity for the ligands used in chromatography rather than a physical association as a heterodimeric combination.

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