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Variation in the expression of Mu-class glutathione *S*-transferase isoenzymes from human skeletal muscle

Evidence for the existence of heterodimers

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The cytosolic glutathione *S*-transferases (GST) from human skeletal muscle were purified by a combination of affinity chromatography and anion-exchange chromatography followed by either chromatofocusing or hydroxyapatite chromatography. Pi-class and Mu-class GST, but not Alpha-class GST, were isolated from muscle. In addition to a Pi-class GST subunit, which exists as a homodimer, this tissue also contains a total of three distinct neutral-type Mu-class GST subunits, which hybridize to form homodimers or heterodimers. The neutral-type subunits are referred to as N_1–N_3 and are defined by the decreasing isoelectric points of the homodimers; GST N_1N_1, N_2N_2 and N_3N_3 have estimated pI values of 6.1, 5.3 and < 5.0 respectively. SDS/PAGE showed that N_1, N_2 and N_3 have M_r values of 26700, 26000 and 26300 respectively. The N_1, N_2 and N_3 subunits are catalytically distinct, with N_1 possessing a high activity for *trans*-4-phenylbut-3-en-2-one and N_2 having high activity with 1,2-dichloro-4-nitrobenzene. In skeletal muscle the expression of the N_1 subunit, but not of N_2 and N_3 subunits, was found to differ from specimen to specimen. The N_1 subunit was absent from about 50% of samples examined, and the purification results from two different specimens are presented to illustrate this inter-individual variation. Skeletal muscle from one individual (M1), which did not express N_1, contained only GST N_2N_2, N_2N_3 and π, whereas the second sample examined (M2) contained GST N_1N_2, N_2N_2 and N_2N_3 as well as GST π. *N*-Terminal amino acid sequence analysis supported the electrophoretic evidence that the N_2 subunit in GST N_1N_2, N_2N_2 and N_2N_3 represents the same polypeptide. The peptides obtained from CNBr digests of N_2 were subjected separately to automated amino acid sequencing, and the results indicate that N_2 is distinct but closely related to the protein encoded by the human Mu-class cDNA clone GTH_4 [DeJong, Chang, Whang-Peng, Knutsen & Tu (1988) Nucleic Acids Res. **16**, 8541–8554]. GST N_2N_2 is probably identical with GST 4 [Board, Suzuki & Shaw (1988) Biochim. Biophys. Acta **953**, 214–217], as over the 24 *N*-terminal residues of GST 4 there is complete identity between the two enzymes. Our data suggest that the GST 1 and GST 4 loci are part of the same multi-gene family.

INTRODUCTION

The glutathione *S*-transferases (GST), a complex group of isoenzymes, function as a part of the phase II drug metabolism response, which provides protection against xenobiotics (Chasseaud, 1979; Ketterer, 1988; Pickett & Lu, 1989). The mammalian cytosolic enzymes are dimeric and can be divided into three classes, which are distinguishable by their physical, structural, catalytic and immunological characteristics (Mannervik, 1985; Hayes & Mantle, 1986*a*). In man these classes were originally described as the basic, near-neutral and acidic forms on the basis of the isoelectric points of the enzymes, but recently these have become more commonly referred to as Alpha-class, Mu-class and Pi-class GST respectively (Ålin *et al.*, 1985). Other workers, who have taken a genetic approach to the classification of these enzymes, have numbered the human GST according to their gene loci, using evidence obtained from zymogram analysis (Board, 1981; Strange *et al.*, 1984). This approach has given rise to the designation GST 1, GST 2 and GST 3, which are the loci that encode enzymes of the Mu-class, Alpha-class and Pi-class GST respectively.

In man, as in other species, the GST in liver have been the most extensively studied. Three hepatic Alpha-class enzymes formed by the combination of two structurally and immunologically distinct subunits termed B_1 and B_2 have been identified (Stockman *et al.*, 1985, 1987; Hayes *et al.*, 1989). The B_1 and B_2 subunits are the products of two independent genes (Rhoads *et al.*, 1987) encoded by the GST 2-1 and GST 2-2 loci (Board, 1981, 1990) respectively. Kamisaka *et al.* (1975) originally described five cationic forms of GST (α, β, γ, δ and ϵ) in human liver. Subsequent studies (Stockman *et al.*, 1985) have shown that the homodimeric enzyme B_1B_1 represents GST ϵ and the heterodimer B_1B_2 is equivalent to GST δ. The relationship of B_2B_2 to these enzymes is less clear; this homodimer probably represents α, β and γ, three GST forms that may have arisen, during purification, from autoxidation of a single gene product (for further discussion see Hayes *et al.*, 1989).

The Mu-class GST in human liver are represented by two well-characterized enzymes termed μ (Warholm *et al.*, 1983) and ψ (Hussey *et al.*, 1986; Singh *et al.*, 1987; Hayes, 1989). These homodimeric proteins are highly homologous (Hayes, 1989), and it has been suggested that they represent allelic variants encoded by the GST 1 locus, which was earlier identified by Board (1981) and Strange *et al.* (1984). The existence of a heterodimeric form

Abbreviation used: GST, glutathione *S*-transferase(s).

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of these enzymes has been described (Faulder *et al.*, 1987); however, so far it has not been purified from human tissue. Between 40 and 45% of individuals fail to express either of these Mu-class GST (Strange *et al.*, 1984; Hussey *et al.*, 1987), and it has been suggested that those lacking μ or ψ, which are the most efficient GST in the detoxification of mutagenic epoxides, may be more susceptible to lung cancer (Seidegård *et al.*, 1986, 1990).

The Pi-class GST isolated from placenta (Howie *et al.*, 1988) is the most thoroughly characterized extrahepatic enzyme. This enzyme is not expressed in hepatocytes, but is found in biliary epithelium, lung and erythrocytes. The available evidence suggests that these Pi-class enzymes, usually termed π, are the products of a single gene (Cowell *et al.*, 1988; Morrow *et al.*, 1989) encoded at the GST 3 locus.

The existence of further extrahepatic GST was first revealed by means of zymogram analysis (Laisney *et al.*, 1984). These enzymes, predominantly expressed in skeletal muscle, heart and brain, which were thought to be the products of additional gene loci termed GST 4, GST 5 and GST 6 (Suzuki *et al.*, 1987), are poorly characterized, and the molecular basis for the existence of these further enzyme forms (i.e. GST 4–6) is unclear. Board *et al.* (1988) have purified an enzyme from human skeletal muscle, called GST 4, that is immunologically related to GST μ and has *N*-terminal amino acid sequence homology with GST μ. Others (Singh *et al.*, 1988) have isolated novel GST from skeletal muscle with *N*-terminal sequences either identical with or closely related to those of GST μ or GST 4.

In the present study we have purified skeletal-muscle GST from several individuals to permit the isoenzymes present to be characterized and to determine whether these enzymes are subject to inter-individual variation.

EXPERIMENTAL

Materials

Chemicals used were all of analytical grade and were readily available commercially. The h.p.l.c. solvents were obtained from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.

Buffers

The pH values quoted are those determined at the temperature at which the buffers were used.

Tissues

Human skeletal-muscle specimens M1 and M2 were obtained less than 16 h *post mortem* from an 84-year-old female and a 60-year-old male respectively. A macroscopic examination at autopsy indicated that there was no evidence of any musculoskeletal abnormality in these individuals. Tissue was obtained from both right and left psoas muscles and stored at −70 °C until used.

Analytical methods

Protein concentrations were determined by the method of Bradford (1976) with the use of a centrifugal fast analyser. SDS/PAGE was carried out by the method of Laemmli (1970) as described elsewhere (Hayes & Mantle, 1986*b*). The resolving gel contained 12% (w/v) polyacrylamide and 0.32% (w/v) *NN'*-methylenebisacrylamide.

Isoelectric focusing was performed in an LKB Multiphor apparatus, as described by the manufacturer (LKB Produkter, Bromma, Sweden), with a broad-range gel (pH 3.5–9.5) in thin-layer 5% (w/v) polyacrylamide. The gel was calibrated with protein pI standards obtained from Pharmacia, Milton Keynes, Bucks., U.K. The method of Habig & Jakoby (1981) was employed for all substrates studied with the exception of cumene hydroperoxide. Peroxidase activity was measured by an adaptation of the method of Wendel (1981) at pH 7.6 with final concentrations of GSH, NADPH and glutathione reductase at 1.0 mM, 0.25 mM and 1 unit/ml respectively.

Enzyme purification

Portions of frozen skeletal-muscle specimen M1 (375 g) and M2 (247 g) were allowed to thaw at room temperature, and 25% (w/v) homogenates were prepared in ice-cold 20 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl and 0.5 mM-dithiothreitol (buffer A). Unless otherwise stated all subsequent steps were performed at 4 °C. The supernatants at 100000 *g*, referred to as cytosol, were passed through plugs of glass-wool before dialysis for 24 h against two changes, each of 5 litres, of buffer A.

M1 and M2 cytosols were then subjected to affinity chromatography on columns (1.6 cm × 30 cm) of *S*-hexylglutathione–Sepharose 6B equilibrated in buffer A. After a washing, with about 800 ml of buffer A, and elution by 5 mM-*S*-hexylglutathione in the same buffer, the bound material was dialysed for 24 h against two changes, each of 2 litres, of 20 mM-Tris/HCl buffer, pH 8.4, containing 0.5 mM-dithiothreitol (buffer B).

Partial resolution of the GST isoenzymes present in the affinity-purified material was achieved by the use of anion-exchange chromatography on Mono Q HR 5/5 with the integrated Pharmacia f.p.l.c. system. This procedure was carried out at room temperature. The Mono Q columns were equilibrated with buffer B (pH 8.0 at 20 °C) at 0.75 ml/min and developed in two stages with linear salt gradients of 0–0.15 M-NaCl in buffer B followed immediately by 0.15–1.0 M-NaCl in buffer B. Fractions eluted from the Mono Q column that contained protein were combined, seven peaks from M1 (P1–P7) and five peaks from M2 (P1, P3 and P5–P7), and analysed by SDS/PAGE before further purification.

The material designated P1, obtained from M2, was dialysed against two changes, each of 2 litres, of 25 mM-Bistris/iminodiacetic acid buffer, pH 7.1, before being chromatofocused, at 20 °C, in the pH range 7–4 on a Mono P HR 5/20 f.p.l.c. column (Pharmacia). Four protein-containing peaks were resolved by this method (P1-A, P1-B, P1-C and P1-D).

The GST activity present in peak P3 from Mono Q, purified from M1 and M2, was resolved into two peaks (P3-A and P3-B) by hydroxyapatite chromatography with a Waters h.p.l.c. system (Waters Associates, Northwich, Cheshire, U.K.). The material present in peak P3 was first dialysed against two changes, each of 2 litres, of 10 mM-sodium phosphate buffer, pH 7.0, containing 0.5 mM-dithiothreitol before application at room temperature to a Bio-Gel HPHT column (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). A 10–350 mM-sodium phosphate gradient, at pH 7.0, was employed to develop the column at a flow rate of 0.5 ml/min.

Immunoblotting

Antisera against purified human GST were raised in New Zealand White rabbits as described previously (Hayes & Mantle, 1986*a*). Western blotting was performed by the method of Towbin *et al.* (1979).

Reverse-phase h.p.l.c.

This was carried out as described previously (Hayes *et al.*, 1989), with a Waters h.p.l.c. system with a μBondapak C_{18} column (Millipore, Harrow, Middx., U.K.). Purified skeletal-muscle GST subunits were resolved with linear gradients of 40–55% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid.

CNBr cleavage

A portion (1.0 mg) of GST isoenzyme P3-B, purified under

reducing conditions, was dialysed extensively against 10 mM-ammonium acetate buffer, pH 7.0, before being freeze-dried. The freeze-dried protein was then subjected to CNBr cleavage as described elsewhere (Hayes *et al.*, 1989). The freeze-dried peptides were redissolved in 1.5 ml of aq. 0.1% (v/v) trifluoroacetic acid and resolved by reverse-phase h.p.l.c. on the μBondapak C_{18} column with a 0–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid.

Amino acid sequencing

Purified skeletal-muscle subunits and the fragments obtained from CNBr cleavage of isoenzyme P3-B were checked for purity on an Applied Biosystems 130 A Microbore Separation System (Applied Biosystems, Warrington, Cheshire, U.K.) before being subjected to automated sequencing on an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyser. For a complete description of these methods see Hayes *et al.* (1989).

RESULTS

Variability in the expression of GST isoenzymes in human skeletal muscle

Analyses of affinity-purified muscle GST by anion-exchange chromatography yielded profiles that differed from specimen to specimen (see Figs. 1*a* and 1*c*). Material from skeletal-muscle specimen M1 was resolved by Mono Q into seven protein-containing pools, two of which (P3 and P6) contained the major GST forms. SDS/PAGE showed that peaks P1 and P2, which did not have GST activity with 1-chloro-2,4-dinitrobenzene as substrate, both contained a single band with a subunit M_r of 28500 (Fig. 1*b*). P3, the first major peak, contained the 28500-M_r polypeptide together with bands that co-migrated with the human placental 24800-M_r GST π standard and with a 26000-M_r standard. The minor peaks P4 and P5 contained polypeptides of the same electrophoretic mobility as that of peak P3; however, peak P5 contained an additional band with an estimated subunit M_r of 22500, which probably represents glyoxylase I (Hayes, 1988). The second major peak (P6) yielded two electrophoretic bands, one of which co-migrated with the 26000-M_r standard and a second diffuse band with a subunit M_r of 26300. This 26300-M_r band was also observed in peak P7, as was the 22500-M_r polypeptide tentatively identified as glyoxylase I.

By contrast, when the affinity-purified GST pool from skeletal-muscle specimen M2 was subjected to the same anion-exchange chromatography step only five peaks were resolved by Mono Q (Fig. 1*c*). The most significant difference between the elution profile of specimen M2 and that obtained from specimen M1 was the increased size of the first peak, P1, and the fact that, unlike peak P1 from the M1 specimen, this pool contained GST activity.

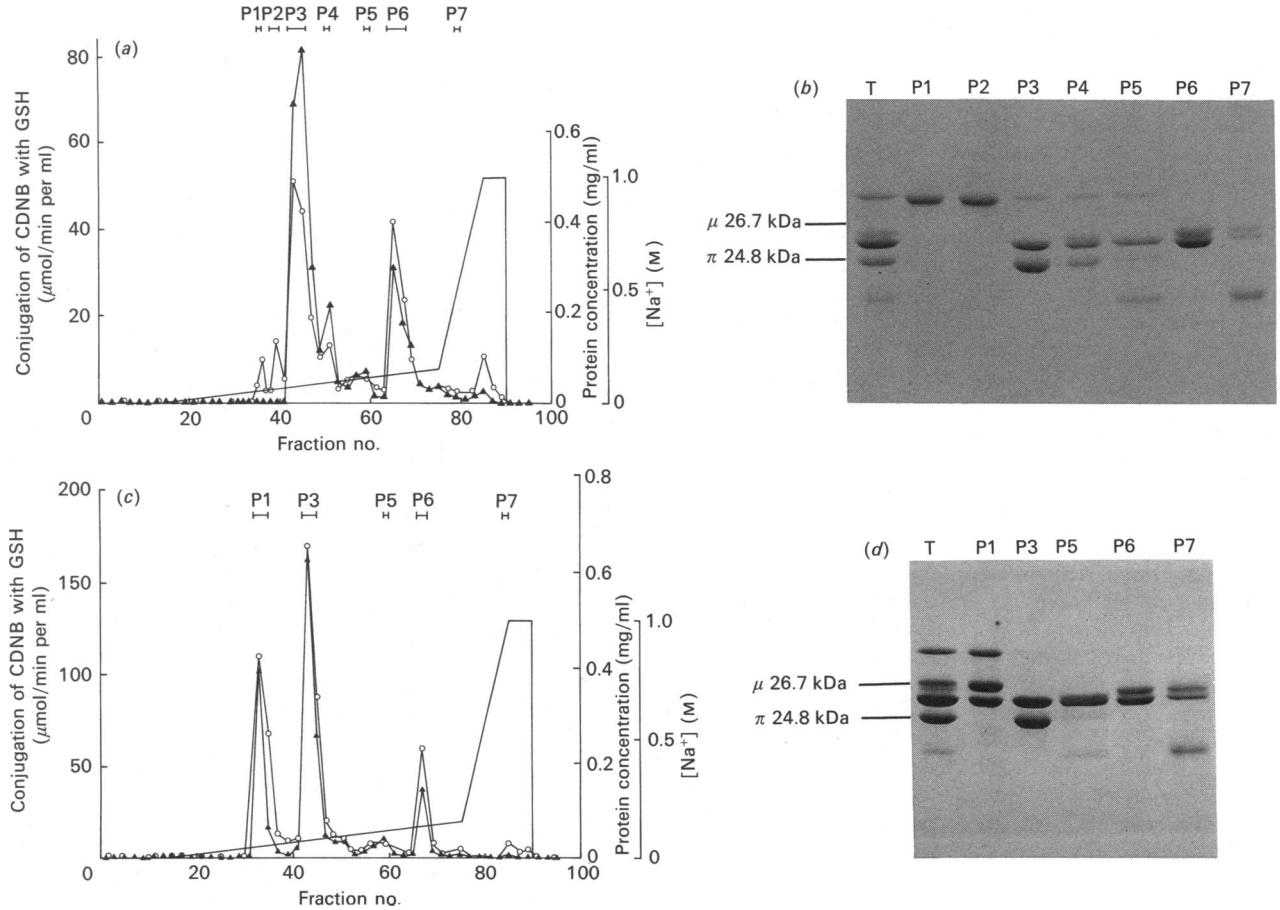

Fig. 1. Resolution of human skeletal-muscle GST by anion-exchange chromatography and SDS/PAGE analysis of individual peaks

S-Hexylglutathione-affinity-purified GST were resolved by anion-exchange chromatography on Mono Q as described in the text. A 0–1.0 M-NaCl gradient was established in two steps as shown by the straight lines. Transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and protein concentration (○) were measured. Panels (*a*) and (*c*) show elution profiles obtained from material purified from specimens M1 and M2 respectively. The pooled fractions, indicated by the horizontal bars, were combined and subjected to electrophoretic analysis as shown in panels (*b*) and (*d*). SDS/PAGE was performed in a 12% (w/v) resolving gel. The positions of the human GST markers μ (M_r 26700) and π (M_r 24800), which were run in parallel, are indicated. The first lanes were loaded with total *S*-hexylglutathione-affinity-purified material (T; 6 μg). The following seven lanes in panel (*b*) and five lanes in panel (*d*) contained the combined fractions from Mono Q (4 μg).

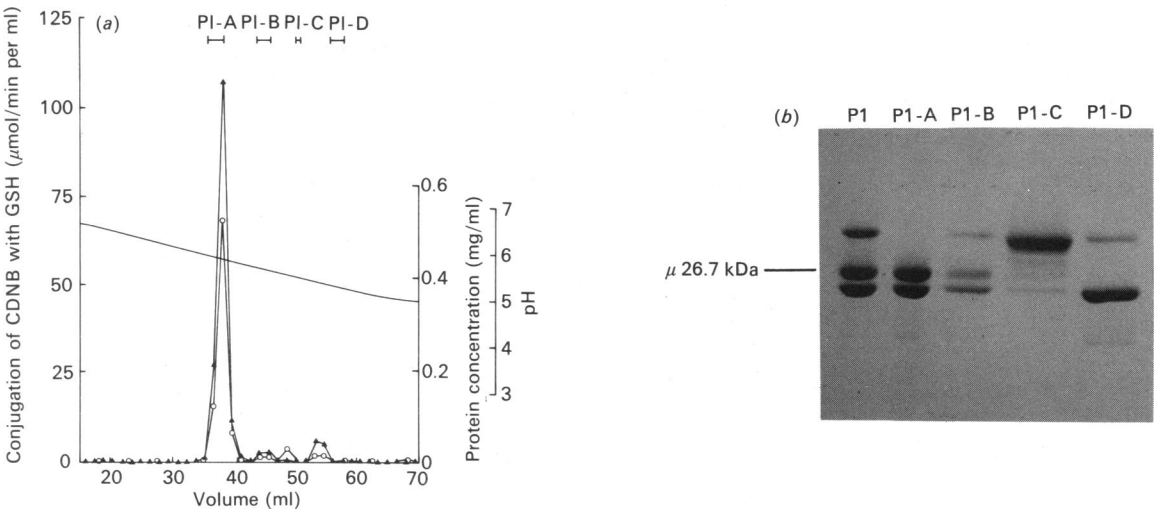

Fig. 2. Chromatofocusing of partially purified skeletal-muscle GST and SDS/PAGE analysis of pooled fractions

Skeletal-muscle GST activity, from specimen M2, purified by *S*-hexylglutathione affinity chromatography and anion-exchange chromatography on Mono Q (P1), was resolved by chromatofocusing on Mono P as described in the text (panel *a*). Transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (▲) and protein concentration (○) were measured, and the pH was monitored (——). Fractions were combined, as indicated by the horizontal bars, and the subunit composition was analysed by SDS/PAGE (panel *b*). The first lane was loaded with the material applied to the chromatofocusing column (P1; 4 μg). The following lanes contained the pools obtained from Mono P, P1-A (4 μg), P1-B (1.5 μg), P1-C (4 μg) and P1-D (3 μg), as shown. The mobility of the human GST marker μ (M_r 26700), which was run in parallel, is indicated.

When examined by SDS/PAGE (Fig. 1*d*), three subunit bands were observed; the largest was the 28500-M_r polypeptide, the second band co-migrated with the human hepatic GST μ standard (M_r 26700) and a third subunit co-migrated with the 26000-M_r standard. Apart from the absence of the polypeptide of M_r 28500, peaks P3, P5, P6 and P7 appeared to have the same subunit compositions as the corresponding peaks on the elution profile obtained from specimen M1. The additional subunit, present only in specimen M2, that co-migrated with GST μ (M_r 26700) was also clearly visible when the affinity-purified total GST pool from specimen M2 was analysed by SDS/PAGE (T in Fig. 1*d*). Hence specimen M1 expressed GST subunits of M_r 24800, 26000 and 26300 whereas specimen M2 expressed not only these three polypeptides but also a GST subunit of M_r 26700.

The GST in muscle specimen M2 that was eluted from Mono Q in peak P1 were purified further by chromatofocusing on Mono P. This column resolved the material recovered in peak P1 into one major peak, P1-A, and three minor peaks, P1-B, P1-C and P1-D (Fig. 2*a*). The protein-containing fractions were combined and their subunit compositions examined by SDS/PAGE (Fig. 2*b*). Peak P1-A, which was eluted from the chromatofocusing column at pH 5.9, contained the two subunits that co-migrated during SDS/PAGE with the liver GST standards of M_r 26000 and 26700. Electrophoresis showed that peak P1-B also contained these two subunits of M_r 26000 and 26700 as well as the polypeptide with M_r 28500. P1-C, the only peak that did not have GST activity, contained the contaminant polypeptide of M_r 28500. Peak P1-D contained trace amounts of the contaminant and the GST subunit with M_r 26000.

Hydroxyapatite h.p.l.c., a technique that separates the human hepatic GST according to their class (Hussey *et al.*, 1986), was employed to resolve the different subunit forms present in peak P3 from both muscle specimens. The profile obtained when material from specimen M2 was applied to this column is shown in Fig. 3(*a*). The first peak, which was eluted on the early part of the phosphate gradient, indicated the presence of Pi-class GST, and the second peak, which was eluted half-way along the gradient, indicated the presence of Mu-class subunits. Analysis by SDS/PAGE (Fig. 3*b*) confirmed that the first peak (P3-A) contained the subunit with the same mobility as GST π, whereas the second peak (P3-B) contained the subunit with an estimated M_r of 26000. Peak P3-B possibly, therefore, contains Mu-class subunits with a greater anodal mobility during SDS/PAGE (M_r 26000) than that of the Mu-class GST μ/ψ isoenzymes of human liver (M_r 26700). The results of the purification of the GST isoenzymes of skeletal muscle are summarized in Table 1.

Identification and characterization of three Mu-class isoenzymes and a Pi-class isoenzyme from human skeletal muscle

Upon analytical isoelectric focusing the GST pools P1-A, P3-A, P3-B and P6 focused into single bands, thereby confirming their purity. Electrophoretic analysis therefore indicates that peaks P3-A and P3-B probably contain homodimeric GST whereas peaks P1-A and P6 contain heterodimeric proteins. Western-blot analysis revealed that the components of peaks P1-A, P3-B and P6 cross-reacted with antisera raised against GST μ but not with antisera raised against either Alpha-class or Pi-class GST. By contrast, the component of peak P3-A was found to cross-react with antibodies to Pi-class GST but not with other antibodies against other GST. These immunochemical data suggest that peaks P1-A, P3-B and P6 contain Mu-class subunits and that peak P3-A contains Pi-class subunits. Together the immunochemical and electrophoretic data suggested the presence of three neutral-type Mu-class GST subunits in human muscle. By designating these as N_1, N_2 and N_3 (according to their apparent pI values) we were able to use a more systematic designation for the muscle enzymes that reflects their quaternary structures. Table 2 summarizes the physical and immunological characteristics of the skeletal-muscle GST; the hepatic GST μ is also included for comparison.

The specific activities of the muscle isoenzymes with a range of substrates are shown in Table 3. All of the enzymes purified from skeletal muscle have high activity with 1-chloro-2,4-dinitrobenzene as a substrate. However, when 1,2-dichloro-4-nitrobenzene or *trans*-4-phenylbut-3-en-2-one was employed considerably different patterns of activity were observed for the

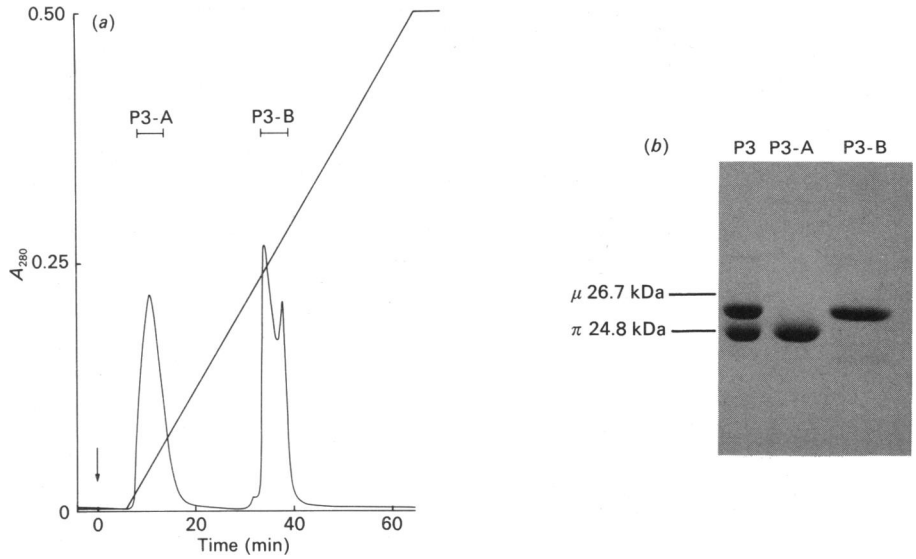

Fig. 3. Hydroxyapatite chromatography of partially purified skeletal-muscle GST isoenzymes and SDS/PAGE of resulting peaks

Enzymes purified from skeletal-muscle specimen M1 by *S*-hexylglutathione affinity chromatography and anion-exchange chromatography on Mono Q (P3) were further resolved by chromatography on Bio-Gel HPHT as described in the text (panel *a*). The column was developed with a linear 10–350 mM-sodium phosphate gradient, indicated by the straight line, and the A_{280} was monitored (——). SDS/PAGE analysis of the combined fractions, indicated by the horizontal bars, is shown in panel (*b*). The mobilities of the human GST markers μ (M_r 26700) and π (M_r 24800) are indicated. The first lane contained the material applied to the hydroxyapatite h.p.l.c. column (P3; 4 μg). The following lanes were loaded with the combined fractions, P3-A (4 μg) and P3-B (4 μg), as shown.

skeletal-muscle enzymes. The homodimeric Mu-class GST N_2N_2 (peak P3-B) and the heterodimeric Mu-class GST N_2N_3 (peak P6) both had activity with 1,2-dichloro-4-nitrobenzene but no detectable activity with *trans*-4-phenylbut-3-en-2-one, unlike the liver enzyme GST μ, where the converse is true. The heterodimeric Mu-class GST N_1N_2 (peak P1-A), however, had activity with both of these substrates. GST μ and GST N_1N_2 were the only Mu-class enzymes to have activity with cumene hydroperoxide. The Pi-class skeletal-muscle enzyme (peak P3-A), like the placental enzyme GST π, had a relatively high activity with ethacrynic acid, but neither GST N_1N_2 nor GST N_2N_2 nor GST N_2N_3 was as active with this substrate.

Subunit separation and *N*-terminal sequence analysis

Before sequence analysis, reverse-phase h.p.l.c. was employed in an attempt to prepare separately the individual subunits present in the skeletal-muscle GST isoenzymes. Unfortunately, the N_1 and N_2 subunits could not be resolved on the μBondapak C_{18} column, but the N_3 subunit was resolved from these two Mu-class polypeptides by reverse-phase h.p.l.c. This technique was also used to purify further GST N_2N_2 (peak P3-B) by removing small residual quantities of the 28500-M_r polypeptide that contaminated certain preparations (Fig. 4). The elution of the two subunits present in GST N_2N_3 (peak P6) from the μBondapak

Table 1. Purification of human skeletal-muscle GST isoenzymes

For experimental details see the text. GST activity was measured with 1-chloro-2,4-dinitrobenzene at 37 °C.

Fraction	Skeletal-muscle specimen M1			Skeletal-muscle specimen M2		
	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)
Cytosol	9007	5419	0.60	9742	4817	0.49
Affinity-chromatography pool	19.8	2245	77.8	19.4	3817	196.7
Mono Q f.p.l.c. pools:						
P1	–	–	–	3.1	631.2	201.0
P3	3.9	933.0	240.5	4.1	1160	283.6
P6	2.7	253.0	92.3	0.9	163.0	187.4
Mono P f.p.l.c. pool:						
P1-A	–	–	–	1.12	152.0	135.7
HPHT h.p.l.c. pools:						
P3-A	0.66	74.0	112.5	0.73	87.6	120.0
P3-B	0.59	100.8	171.1	1.14	243.0	213.1

Table 2. Physical and immunochemical properties of skeletal-muscle GST isoenzymes

Preparations of antisera were as described in Hayes *et al.* (1983) (GST B_1B_1), Hayes (1989) (subunit N_1 or GST μ) and Howie *et al.* (1988) (GST π). Anti-(subunit N_2) IgG was prepared as described in the Experimental section using N_2 subunits, purified by reverse-phase h.p.l.c., as immunogen.

Enzyme	Preparation designation	Subunit M_r	pI value	Cross-reactivity with antisera against: B_1B_1	N_1	N_2	π
N_1N_1 (μ)*	–	26700	6.10	–	+++	+++	–
N_1N_2	P1-A	{26700 / 26000}	5.65	–	+++	+++	–
				–	++	+++	–
N_2N_2	P3-B	26000	5.30	–	++	+++	–
N_2N_3	P6	{26000 / 26300}	5.00	–	++	+++	–
				–	+	–	–
π	P3-A	24700	4.70	–	–	–	+++

* Data from Hayes (1989)

Table 3. Specific activities of skeletal-muscle GST isoenzymes

For experimental details see the text. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one; EA, ethacrynic acid; CuOOH, cumene hydroperoxide.

Enzyme	Preparation designation	Specific activity (μmol/min per mg at 37 °C) CDNB	DCNB	tPBO	EA	CuOOH
N_1N_1 (μ)*	–	261	0	0.22	0.08	0.22
N_1N_2	P1-A	136±3	2.67±0.04	0.26±0.04	0.41±0.13	0.17±0.01
N_2N_2	P3-B	171±7	1.91±0.32	0	0.33±0.04	0
N_2N_3	P6	92±3	1.61±0.2	0	0.27±0.01	0
π	P3-A	113±1	0.25±0.06	0	0.78±0.01	0

* Data from Hayes (1989).

C_{18} column is also shown in Fig. 4. The major peak, which was eluted at between 37 and 41 min, contained a polypeptide of M_r 26000, whereas the two minor peaks, which were eluted at 50 min and 56 min, both contained subunits of M_r 26300. SDS/PAGE analysis of the GST subunits that were isolated by this technique is shown in Fig. 5.

N-Terminal sequence analysis was undertaken to help establish the relationship between the muscle GST and provide evidence that the polypeptide of M_r 26000 that was found in the different Mu-class enzymes, and that we have designated N_2, is indeed a common subunit. The results of these analyses are shown in Fig. 6. The subunits of M_r 26000 present in the GST N_2N_2 homodimer

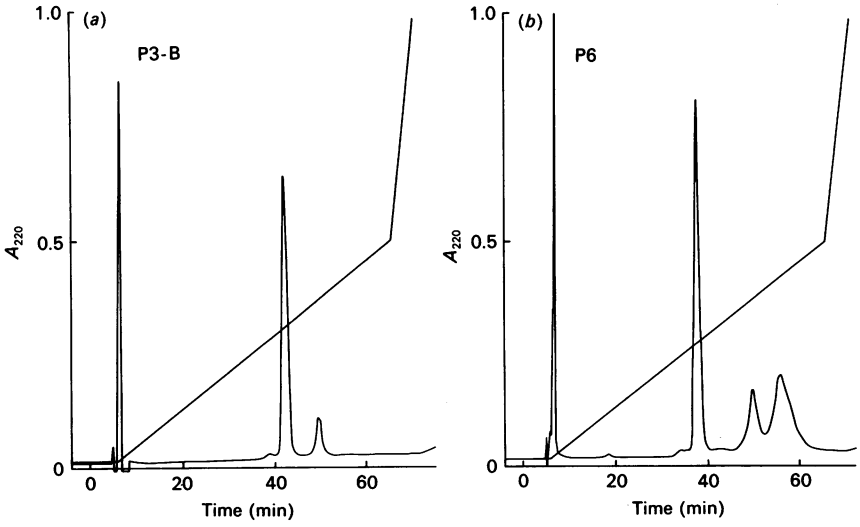

Fig. 4. Reverse-phase h.p.l.c. of human skeletal-muscle GST subunits

Skeletal-muscle GST pools P3-B (*a*) and P6 (*b*) were subjected to reverse-phase h.p.l.c. on a Waters μBondapak C_{18} column (10 μm particle size; 0.39 cm × 30 cm). The column was developed with a 40–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid in two stages as shown by the straight lines. The A_{220} of the eluate was monitored continuously (——).

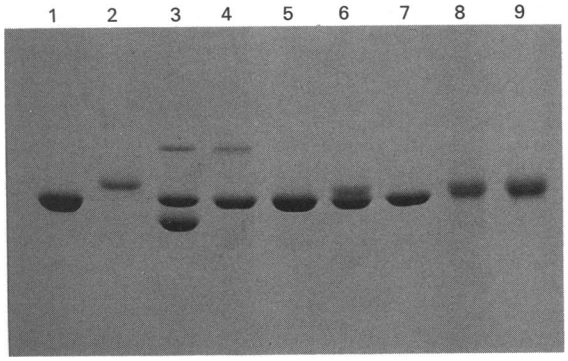

Fig. 5. SDS/PAGE analysis of Mu-Class subunits isolated from human skeletal muscle

SDS/PAGE was performed by using the method of Laemmli (1970). The resolving gel contained 12% (w/v) polyacrylamide and 0.32% (w/v) *NN'*-methylenebisacrylamide. The GST subunits purified on reverse-phase h.p.l.c. were analysed as follows. Lane 1 contained the subunit marker B_1B_2 (M_r 26000) and lane 2 contained the subunit marker μ (M_r 26700). Lane 3 was loaded with pool P3 obtained after anion-exchange chromatography on Mono Q of affinity-purified material (4 μg). Lane 4 contained pool P3-B purified on hydroxyapatite h.p.l.c. (4 μg). Lane 5 contained the GST subunit N_2 isolated from pool P3-B after reverse-phase h.p.l.c. Lane 6 was loaded with pool P6 purified by affinity chromatography and anion-exchange chromatography on Mono Q (4 μg), lane 7 contained the GST subunit N_2 isolated from P6, and lanes 8 and 9 contained the N_3 subunits resolved on reverse-phase h.p.l.c. from P6.

and the GST N_2N_3 heterodimer were shown to have identical *N*-terminal sequences over the first ten residues. Although the subunits in GST N_1N_2 could not be separated on the μBondapak C_{18} column it was possible to interpret the sequence data obtained from the heterodimeric protein over most of the residues analysed. The direct sequencing of GST N_1N_2 yielded unambiguous assignments from cycles 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14, but at residues 3, 8 and 15 the Sequenator found two amino acids, namely threonine or isoleucine, asparagine or aspartic acid and serine or alanine. When these sequences were compared with those of human Mu-class enzymes described in the literature, it was apparent that one sequence could be attributed to that of human liver GST μ or GST ψ (Ålin *et al.*, 1985; Hayes, 1989) and the other was identical with the 24 residues published for the skeletal-muscle enzyme GST 4 (Board *et al.*, 1988). These data suggest that the subunit of M_r 26000, designated N_2, in GST N_1N_2, N_2N_2 and N_2N_3 represents the same polypeptide. The N_3 subunit that was purified by reverse-phase h.p.l.c. was found to possess a blocked *N*-terminus and could not be sequenced directly.

Comparison of the amino acid sequences of human Mu-class GST isoenzymes

The protein sequence information obtained from the muscle Mu-class enzyme GST N_2N_2 has enabled the comparison of 52% of its primary structure with that of other human Mu-class enzymes reported in the literature. Four of the peptides, CNBr-2, CNBr-5, CNBr-6 and CNBr-7, were subjected to automated amino acid sequencing. In Fig. 7 the amino acid sequences obtained from these fragments, together with the *N*-terminal sequence of subunit N_2 (peptide CNBr-1), are shown aligned with the homologous sequences deduced from the human hepatic cDNA clone GTH_4, reported by DeJong *et al.* (1988), and the sequences deduced from the human Mu-class genomic clones, GST mu2 and GST mu3, described by Taylor *et al.* (1990).

Comparison of the *N*-terminal sequence of GST N_2N_2 (peptide CNBr-1) with that of the GTH_4 clone reveals the three amino acid differences found at positions 3, 8 and 15 predicted from the *N*-terminal sequence analysis of GST μ and GST ψ discussed above. This is followed by a region of complete identity between the two sequences from residue 16 to residue 38. The peptide CNBr-2, which was eluted from the μBondapak C_{18} column at 44.5 min, was subjected to 45 automated Edman degradation cycles, allowing identification of residues 34–78. Over this region

Enzyme	Pool	Subunit(s) examined	Residue 1 5 10 15 20
N_1N_1 (μ)*		N_1	P M I L G Y W D I R G L A H A I R L L L E Y T D
N_1N_2	P1-A	N_1+N_2	P M I/T L G Y W D/N I R G L A H A/S
N_2N_2	P3-B	N_2	P M T L G Y W N I R
N_2N_3	P6	N_2	P M T L G Y W N I R G L A H S I R L L L E Y T D
N_2N_3†	P6	N_3	-
GST 4‡			P M T L G Y W N I R G L A H S I R L L L E Y T D

Fig. 6. *N*-Terminal amino acid sequences of GST from human skeletal muscle

Purified skeletal-muscle GST enzymes N_1N_2, N_2N_2 and N_2N_3 were subjected to automated *N*-terminal amino acid sequencing as described in the text. *Data from Hayes (1989). †*N*-Terminus blocked. ‡Data from Board *et al.* (1988).

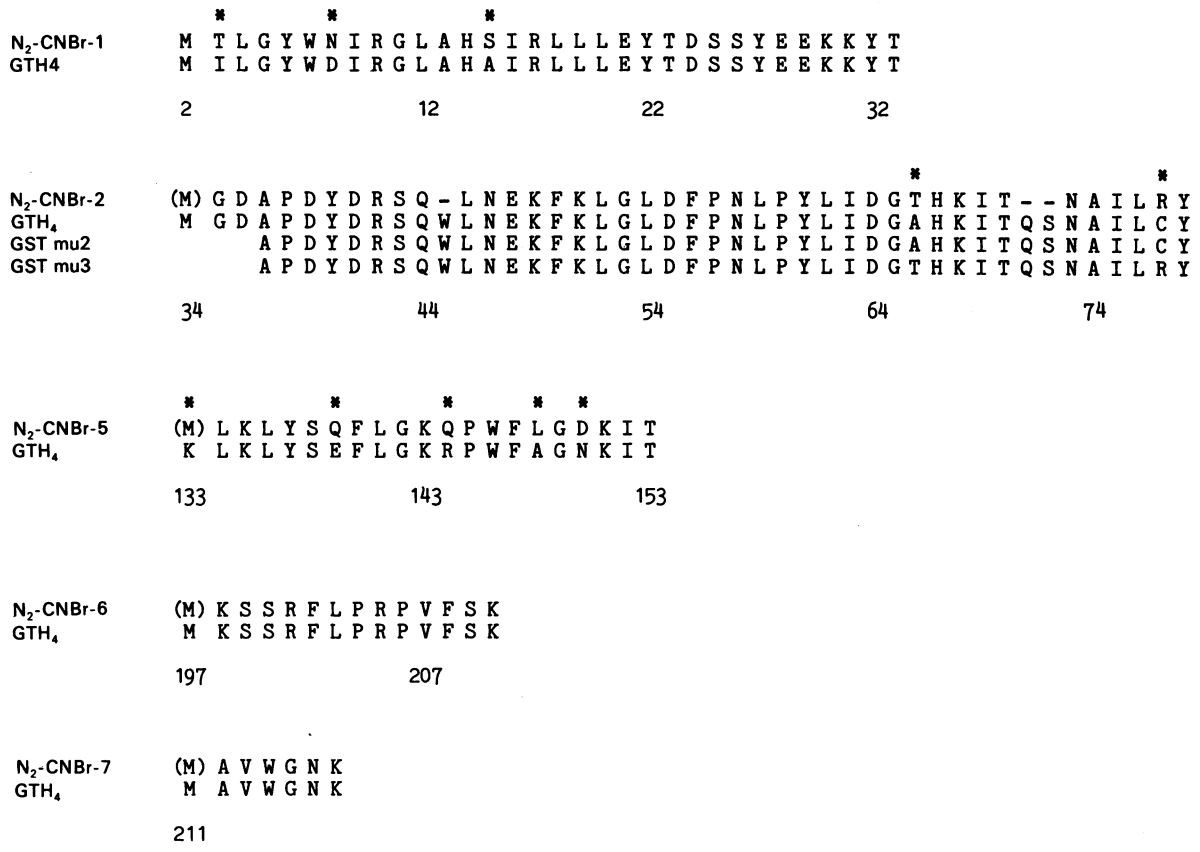

Fig. 7. Comparison of the primary structure of the N_2 subunit with other human Mu-class GST

The *N*-terminal amino acid sequence (CNBr-1) and the sequences of the CNBr-derived peptides from subunit N_2 are aligned for comparison with the homologous sequences deduced from the cDNA GTH_4 (DeJong *et al.*, 1988) and the available homologous sequences deduced from the genomic clones GST mu2 and GST mu3 (Taylor *et al.*, 1990). Putative methionine residues are in parentheses and a dash indicates where no residue could be identified with confidence. An asterisk (*) indicates amino acid residues that differ between the sequences.

of the protein amino acid sequence information on the genomic clones GST mu2 and GST mu3 is available for comparison with subunit N_2. Amino acid differences were apparent between the sequence of the N_2-subunit peptide CNBr-2 and those of the proteins encoded by clones GTH_4 and GST mu2. At position 66 the N_2 subunit contains threonine whereas the proteins encoded by clones GTH_4 and GST mu2 contain alanine, and at position 77 the N_2 subunit possesses arginine whereas the proteins encoded by clones GTH_4 and GST mu2 both contain cysteine. However, when the sequence of N_2-subunit peptide CNBr-2 is compared with that of the protein encoded by clone GST mu3 it is apparent that the two sequences correspond exactly over the region where sequence data on this genomic clone are available (i.e. residues 37–78).

Residues 133–153 of subunit N_2 were identified by sequencing the peptide, CNBr-5, that was eluted at 51 min from the μBondapak C_{18} column. Five amino acid substitutions were present between the sequence of N_2-subunit peptide CNBr-5 and that of the protein encoded by clone GTH_4. At position 133 a methionine residue, which has provided a CNBr-cleavage site, can be assigned to subunit N_2, whereas the protein encoded by clone GTH_4 contains a lysine residue. The other four amino acid changes between N_2-subunit peptide CNBr-5 and the protein encoded by clone GTH_4, at positions 139, 144, 148 and 150, were as follows: glutamine to glutamic acid, glutamine to arginine, leucine to alanine and aspartic acid to asparagine respectively. N_2-subunit peptides CNBr-6 and CNBr-7, which were eluted at 30 min and 23 min from the reverse-phase column, contained residues 198–210 and residues 212–217 respectively. No differences were apparent between this *C*-terminal region of the N_2 subunit and the homologous region of the protein encoded by clone GTH_4.

DISCUSSION

This paper describes the purification of a total of four GST isoenzymes from human skeletal muscle. Three of these were Mu-class GST and the remaining enzyme was a Pi-class GST. No Alpha-class enzymes were isolated from any of the specimens of human muscle. The Mu-class, or neutral, enzymes were shown to comprise three distinct subunits, which we have designated N_1, N_2 and N_3 according to their decreasing pI values. These subunits can hybridize, and we have described the muscle GST by their quaternary structure as N_1N_2, N_2N_2 and N_2N_3. During the present study a N_1N_3 heterodimer has not been recovered, and although its existence seems probable we can offer no explanation for its absence from human muscle. The N_1 subunit was found in only about half of the muscle specimens examined, whereas N_2 and N_3 were recovered in all specimens. Because of the variation in the expression of N_1, the N_1N_2 heterodimer was absent from some of the enzyme preparations.

GST N_1N_2, N_2N_2 and N_2N_3 possess distinct catalytic properties. The N_1N_2 heterodimer was found to possess the greatest activity of all the muscle GST for *trans*-4-phenylbut-3-en-2-one, indicating that the N_1 subunit is responsible for catalysing its conjugation with GSH. All these GST were active with 1,2-

dichloro-4-nitrobenzene, which strongly implicates the N_2 subunit in the metabolism of this compound. No substrate was identified that could serve as a marker for the N_3 subunit. It may be noted that the substrate-specificities of the N_1N_2, N_2N_2 and N_2N_3 muscle enzymes are significantly different from the well-characterized hepatic Mu-class enzyme, GST μ (Table 3). When one examines the activities of these enzymes for 1,2-dichloro-4-nitrobenzene and *trans*-4-phenylbut-3-en-2-one, both diagnostic substrates for the Yb-type subunits in the rat, it is apparent that GST N_2N_2 has a similar substrate-specificity to that of the rat enzymes Yb_1Yb_1 and YnYn, whereas the substrate preference of GST μ is more closely similar to that of rat GST Yb_2Yb_2 (Hayes, 1986).

A modest decrease in the specific activity of the purified GST isoenzymes, with 1-chloro-2,4-dinitrobenzene as substrate, is apparent towards the end of the purification procedure (Table 1). Having examined the data from five skeletal-muscle preparations, we have observed a relatively large amount of variability in the specific activities obtained for the purified Mu-class isoenzymes GST N_1N_2 and GST N_2N_2, whereas the Pi-class enzyme has a specific activity that varies little from preparation to preparation. The basis for this observation is uncertain, but it does not appear to be due to proteolysis, as neither the M_r nor the immunochemical properties of GST N_1N_2 or GST N_2N_2 change during the purification. It therefore seems probable that either an aging effect or the removal of an activator during purification is causing a loss in the enzymic activity of these Mu-class GST in certain preparations. In this context it is noteworthy that certain rat Mu-class GST are labile, and Kispert *et al.* (1989) have proposed that the thiol status of the cysteine residue at position 184 in rat subunit 11 (Yo) is responsible for this property in this particular GST.

The muscle Mu-class GST have different isoelectric points, and their pI values are helpful aids to the identification of the various isoenzymes; GST N_1N_2, N_2N_2 and N_2N_3 have isoelectric points of 5.65, 5.30 and 5.00 respectively. However, because of the closeness of these pI values it is unwise to identify GST solely on the basis of isoelectric-focusing results. The subunits that the Mu-class GST comprise can be resolved conveniently, and identified, by SDS/PAGE. With this method the N_1, N_2 and N_3 subunits have estimated M_r values of 26700, 26000 and 26300 respectively. It is important to recognize that the N_2 subunit and the Alpha-class B_1/B_2 subunits co-migrate during SDS/PAGE and that it is therefore essential to use immunochemical methods to help discriminate between these subunits.

No cross-reactivity was observed between subunits N_1, N_2 and N_3 and antibodies against either the Alpha-class GST B_1B_2 or the Pi-class GST π. All the N-type subunits were found to cross-react with antiserum raised against hepatic GST μ, and therefore, although the N_2 and B_1/B_2 subunits co-migrate during SDS/PAGE, they can be discriminated by Western-blot analysis. However, our data also suggest that the individual N-type subunits can be distinguished by immunochemistry, but that to achieve identification the choice of antiserum is important. The degree of cross-reactivity towards GST μ antibodies was found to vary from subunit to subunit. Whereas the N_1 subunit cross-reacted strongly with this antiserum, the N_2 subunit showed a significantly weaker reactivity, giving a moderate signal in the Western-blot assay and less than 10% cross-reactivity in the GST μ radioimmunoassay (A. F. Howie, A. J. Hussey, J. D. Hayes & G. J. Beckett, unpublished work). By contrast, the N_3 subunit was even less reactive than the N_2 subunit towards anti-(GST μ) antibodies, yielding only a weak signal in the Western-blot assay. We have also raised antibodies against the N_2 subunit and found that, although these cross-react with the N_1 subunit, they do not exhibit reactivity with the N_3 subunit.

N-Terminal amino acid sequencing suggested that subunits N_1, N_2 and N_3 are genetically separate. Although we were able to analyse the purified N_2 and N_3 subunits directly, we were unable to resolve by reverse-phase h.p.l.c. the constituent subunits of GST N_1N_2 and therefore the sequence data for subunit N_1 were derived from the direct analysis of the N_1N_2 heterodimer. Comparison between subunits N_1 and N_2 showed differences at residues 3, 8 and 15; at these positions subunit N_1 appeared to contain isoleucine, aspartic acid and alanine whereas subunit N_2 was found to contain threonine, asparagine and serine. By contrast, the N_3 subunit possesses a blocked *N*-terminus, but unfortunately paucity of material prevented us from undertaking a preparative CNBr digest that could have allowed us to obtain sequence data.

Comparison of the *N*-terminal sequence data for subunit N_1 with the primary structure of the subunits of hepatic GST μ suggests that they are identical. This conclusion is supported by the immunochemical results, the observation that subunit N_1 has a high activity for *trans*-4-phenylbut-3-en-2-one and the inter-individual variability in the expression of the N_1 subunit. Inspection of the sequence data for the N_2 subunit compared with the cDNA clone encoding GST μ (i.e. N_1N_1) shows that they are genetically distinct; differences in primary structure were noted at residues 3, 8, 15, 66, 77, 133, 139, 144, 148 and 150. Moreover, comparison of these data for the N_2 subunit with the *N*-terminal sequence for GST-4 (Board *et al.*, 1988) suggests that subunits N_2 and GST-4 are probably the same. The amino acid sequence analysis of subunit N_2 also allowed comparison with the genomic human Mu-class clones of Taylor *et al.* (1990). This indicated that N_2 represents the subunit encoded by clone GST mu3. The fact that the N_3 subunit possesses a blocked *N*-terminus is itself an interesting observation, as in the rat the only Mu-class GST subunit that has a blocked *N*-terminus is Yo (Hayes, 1988; Kispert *et al.*, 1989), the Yb_1, Yb_2, Yn_1 and Yn_2 subunits having *N*-termini that are amenable to direct sequence analysis. It is therefore possible that subunit N_3 is the homologue of the rat Yo subunit. It is also worth noting that the Mu-class homodimer GST ϕ of pI 4.6, which Stockman & Hayes (1987) found in only one of 20 human liver specimens, may represent N_3N_3 as it also possessed a blocked *N*-terminus.

We have demonstrated the ability of the N_2 subunit to hybridize with other Mu-class subunits. The existence of a heterodimeric protein formed by the combination of the subunits encoded by the GST 1 locus (i.e.N_1) and the GST 4 locus (i.e. N_2) was first predicted, by using zymogram analysis, by Laisney *et al.* (1984). This finding, however, was not confirmed when similar studies were performed by Suzuki *et al.* (1987). The isolation and characterization, described in the present paper, of the N_1N_2 heterodimer supports the earlier work of Laisney *et al.* (1984), and the purification of the N_2N_3 heterodimer represents new information abut the multiplicity of the human Mu-class GST family. It is of interest that the chromosome mapping experiments performed by several workers have located human Mu-class genes on both chromosome 1 (DeJong *et al.*, 1988) and chromosome 3 (Islam *et al.*, 1989).

Two hepatic human Mu-class homodimers, GST μ and GST ψ, have been described (Hussey *et al.*, 1986; Hayes, 1989). These enzymes represent allelic variants and possess closely similar catalytic activities and identical *N*-terminal amino acid sequences (up to 50 residues), but differ in their isoelectric points, with GST μ and GST ψ having pI values of 6.1 and 5.5 respectively. As GST μ/ψ appear to comprise N_1-type subunits, we propose that GST μ be designated $N_1^aN_1^a$ and GST ψ be designated $N_1^bN_1^b$. On the basis of respective pI values of 6.1, 5.5 and 5.3 for $N_1^aN_1^a$, $N_1^bN_1^b$ and N_2N_2, it is probable that the N_1N_2 heterodimer of pI 5.65 described in the present study represents

$N_1{}^aN_2$; it is expected that the isoelectric point of the $N_1{}^bN_2$ heterodimer would be approx. 5.4. This difference in the pI value of the allelic variants of N_1 is an important reason why extreme caution should be exercised in identifying these enzymes solely by isoelectric focusing.

The acidic enzyme, peak 3-A, which was present in large quantities in skeletal muscle of all individuals included in the study, has similar physical and catalytic properties to those of the Pi-class enzyme found in human placenta. Other workers have also observed an enzyme, or enzymes, similar to the placental GST π in skeletal muscle (Board *et al.*, 1988; Singh *et al.*, 1988). Awasthi and co-workers (Singh *et al.*, 1988) found two acidic GST isoenzymes, termed GST 4.8 and GST 4.5, that have identical *N*-terminal amino acid sequences to each other and to that of human placental GST μ. One of these enzymes, GST 4.5, was present in only two of six skeletal muscles studied, and these workers therefore proposed the existence of a polymorphism at the GST 3 locus. Although we have not observed any difference in the Pi-class GST from different specimens of skeletal muscle, it should be recognized that hybridization *in situ* has localized the gene encoding Pi-class GST in man to both chromosomes 11 and 12 (Board *et al.*, 1989). The possibility therefore remains that two GST π isoenzymes exist, but this question remains to be resolved. In this context, it is interesting that the amino acid sequence of fatty acid ethyl ester synthetase from human myocardium has been reported to be highly similar to, but not identical with, that of the placental GST π (Bora *et al.*, 1989). On the basis of the data of Ålin *et al.* (1985) and Bora *et al.* (1989) it is apparent that over the 23 *N*-terminal residues of fatty acid ethyl ester synthetase and placental GST π two amino acid differences exist at residues 1 (alanine→proline) and 15 (lysine→alanine). There is therefore evidence that two separate Pi-class GST are expressed in man, but it is unclear how these relate to the GST 4.5 and GST 4.8 forms described by Singh *et al.* (1988).

From the results presented here it is clear that there are at least one Pi-class and three Mu-class subunits expressed in skeletal muscle. Evidence has been provided that the three Mu-class subunits combine to form homodimeric or heterodimeric proteins and that one of these subunits is absent from the muscle of certain individuals. The characterization of the N_1, N_2 and N_3 subunits has provided a valuable insight to the molecular basis for the multiplicity of Mu-class GST in man.

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