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Identification of a novel glutathione transferase in human skin homologous with Class Alpha glutathione transferase 2-2 in the rat

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Six forms of glutathione transferase with pI values of 4.6, 5.9, 6.8, 7.1, 8.5 and 9.9 have been isolated from the cytosol fraction of normal skin from three human subjects. The three most abundant enzymes were an acidic Class Pi transferase (pI 4.6; apparent subunit M_r 23000), a basic Class Alpha transferase (pI 8.5; apparent subunit M_r 24000) and an even more basic glutathione transferase of Class Alpha (pI 9.9; apparent subunit M_r 26500). The last enzyme, which was previously unknown, accounts for 10–20% of the glutathione transferase in human skin. The novel transferase showed greater similarities with rat glutathione transferase 2-2, another Class Alpha enzyme, than with any other known transferase irrespective of species. The most striking similarities included reactions with antibodies, amino acid compositions and identical *N*-terminal amino acid sequences (16 residues). The close relationship between the human most basic and the rat glutathione transferase 2-2 supports the classification of the transferases previously proposed and indicates that the similarities between enzymes isolated from different species are more extensive than had been assumed previously.

INTRODUCTION

Glutathione transferase (EC 2.5.1.18) is known to occur in multiple forms in various organisms (for a review, see Mannervik, 1985). Rat, mouse and human tissues contain numerous variants of the enzyme, the relationships of which have been incompletely understood. Considerable clarification was achieved by the recent finding that the well-characterized mammalian glutathione transferases can be divided into three distinct species-independent classes, named Alpha, Mu and Pi (Mannervik *et al.*, 1985). The classification was based on several criteria, including substrate specificities, inhibition characteristics, reactions with specific antibodies and amino acid sequence analyses. The enzymes of Class Mu and Class Pi all had free *N*-terminal amino acids, and it was possible to demonstrate significant amino acid sequence homologies among the members of a class. The members of Class Alpha so far analysed, with the exception of two enzymes containing rat subunit 2, all appear to have blocked *N*-termini. Therefore, apart from rat subunit 2, the only known amino acid sequences of Class Alpha enzymes are for two variants of rat subunit 1, both deduced from cloned cDNA nucleotide sequences (Pickett *et al.*, 1984; Lai *et al.*, 1984). For the classification to be rigorously established, it is therefore of great importance to obtain structural data for Class Alpha transferases from species other than the rat. The present investigation shows that human skin contains Class Alpha transferases and a Class Pi transferase. One of the Class Alpha transferases has not previously been described in human tissues. This glutathione transferase has a free *N*-terminal amino acid residue and shows identity with the primary structure of rat glutathione transferase 2-2 in the *N*-terminal region.

MATERIALS AND METHODS

Procedures for measuring the glutathione transferase activity with different substrates, the sensitivities to inhibitors, and other characteristics of the enzymes were essentially those described previously (Ålin *et al.*, 1985).

Amino acid sequence analysis was made with a gas-phase sequencer (Applied Biosystems). A sample of the most basic transferase was analysed for 16 cycles. The phenylthiohydantoin derivatives were identified and quantified by h.p.l.c. (Zimmerman *et al.*, 1977).

Human skin was obtained fresh from amputated legs and appeared normal by morphological criteria. Three specimens from male Caucasian subjects aged 55–61 years were used; the analysis of the sample from a 55-year-old donor is described below. This tissue was dissected free from adipose tissue and 77.5 g was cut into small pieces and homogenized in 4 vol. of 10 mM-potassium phosphate, pH 7.2, containing 1 mM-EDTA and 2 mM-dithiothreitol (buffer A), in a Waring blender for 1 min at 15000 rev./min. The homogenate was centrifuged at 13000 *g* for 30 min, and the resulting supernatant fraction was further centrifuged at 105000 *g* for 60 min. The cytosol fraction was collected by use of a syringe in order to avoid contamination by the lipid layer at the top of the centrifuge tubes. The material thus obtained was filtered through cotton to remove residual

Abbreviations used: individual human glutathione transferases are denoted by Greek letters (e.g. α, π), whereas species-independent classes (Mannervik *et al.*, 1985) have been designated by the names of Greek letters spelled out in full (e.g. Class Alpha, Class Pi).

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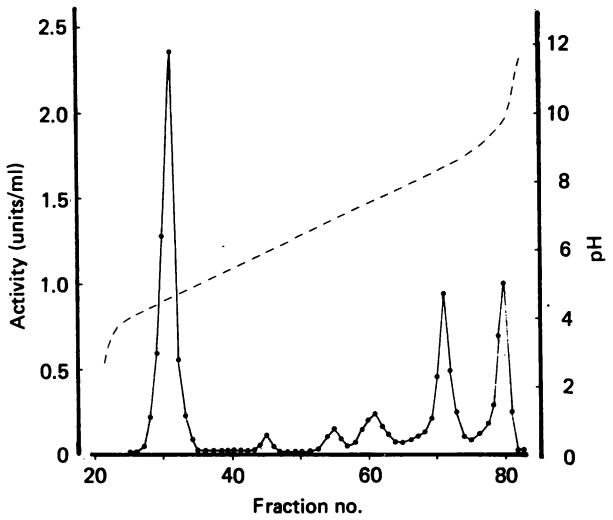

Fig. 1. Separation of cytosolic glutathione transferases from human skin by isoelectric focusing in a 110 ml column

Fractions (1.3 ml each) were collected and the enzymic activity with 1-chloro-2,4-dinitrobenzene (●) as well as the pH (----) were measured.

lipid material. The filtrate (270 ml) was applied to a GSH–Sepharose 6B affinity column (10 cm × 1 cm) (Simons & Vander Jagt, 1977) equilibrated with buffer A. The column was washed with several bed volumes of the same buffer supplemented with 50 mM-KCl, and glutathione transferases were eluted with 50 mM-Tris/HCl, pH 9.6, containing 5 mM-GSH. The enzyme-containing fractions were pooled (93 ml), concentrated to 7.8 ml by ultrafiltration on a Diaflo PM10 filter and dialysed against several columes of buffer A. A sample (7.0 ml) was applied to an isoelectric-focusing column (110 ml; LKB Produkter, Stockholm, Sweden) containing 1% Ampholine, pH 3.5–10, plus 1% Ampholine, pH 9–11, in a 0–40% (w/v) sucrose density gradient. After focusing for 72h at a final voltage of 700 V (4 °C), the contents of the column were eluted and collected in 1.3 ml fractions. The most active fractions of the three major glutathione transferase peaks thus separated were concentrated by ultrafiltration and dialysed against buffer A. The total activity of the three forms of glutathione transferase collected for further analysis represented 40% of the activity applied to the isoelectric-focusing column.

All operations in the purification procedure were performed at 4 °C.

RESULTS

Purification of glutathione transferases from human skin

Fig. 1 shows the distribution of glutathione transferase activity as measured with 1-chloro-2,4-dinitrobenzene after isoelectric focusing of a sample purified by affinity chromatography. Six forms of the enzyme, with pI values of 4.6, 5.9, 6.8, 7.1, 8.5 and 9.9, were resolved. Only the major forms, in the first, fifth and sixth activity peaks, contained material sufficient for further characterization. In addition to the glutathione transferases isolated by the affinity chromatography, enzyme corresponding to 3% of the total activity passed unadsorbed through the affinity matrix.

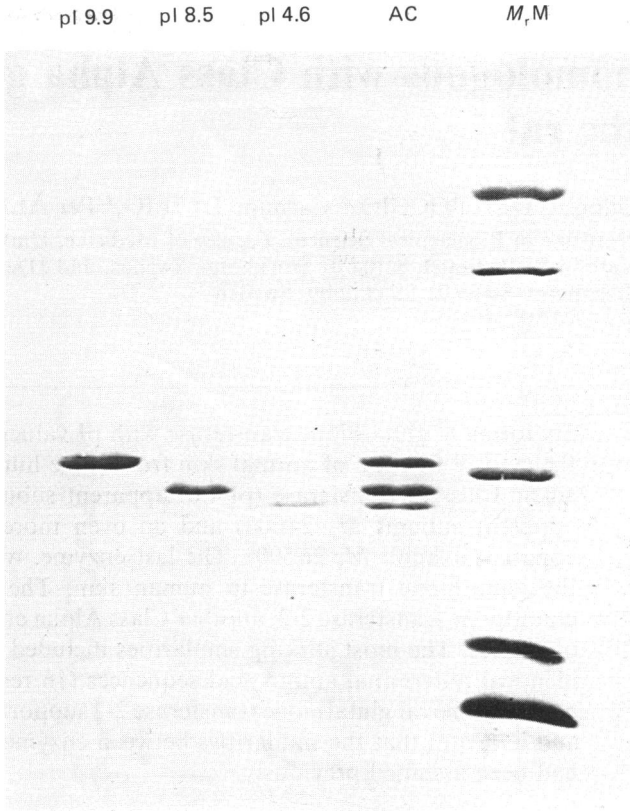

Fig. 2. SDS/polyacrylamide-slab-gel electrophoresis of the purified major forms of glutathione transferase in human skin

The lanes from left to right represent the homogeneous transferases with pI values 9.9, 8.5 and 4.6, followed by the unresolved mixture of transferases purified by affinity chromatography (AC) and M_r markers (M_rM) (12500, 13000, 25000, 45000 and 64000).

Characterization of the major enzyme forms

The most acidic glutathione transferase indicated in Fig. 1 can be referred to Class Pi on the basis of high specific activity with the substrate ethacrynic acid, apparent subunit M_r (23000) (Fig. 2), pI (4.6) and amino acid composition (Table 1) (cf. Guthenberg & Mannervik, 1981).

The fifth enzyme form (pI8.5) can be identified as a Class Alpha transferase on the basis of high specific activity with cumene hydroperoxide, pI, amino acid composition (Table 1) and an apparent subunit M_r of 24000 intermediate between the values for transferases π and μ (Kamisaka *et al.*, 1975; Warholm *et al.*, 1983).

The sixth enzyme form (pI 9.9) by similar criteria was classified as a Class Alpha transferase. Since this enzyme appears not to have been characterized before, its properties are described more extensively below.

For further support for the classification of the major forms, Ouchterlony double-diffusion experiments were performed with antibodies raised against the human glutathione transferases α–ϵ, μ and π (Warholm *et al.*, 1983) as well as against rat glutathione transferases 1-1 and 2-2 (Ålin *et al.*, 1985). The most acidic enzyme from skin gave a precipitin reaction with anti-transferase π antibodies, and the fifth enzyme form (at pH 8.5) reacted with antibodies against a mixture of human Class

Table 1. Amino acid analyses of glutathione transferase from human skin

Amino acid analyses were performed after hydrolysis at 110 °C in 6 M-HCl for 24, 48 and 72 h. Compositions are based on the following M_r values for the different transferases: 46000 (pI 4.6), 48000 (pI 8.5) and 53000 (pI 9.9). Values are averages, except those for serine and threonine, which were extrapolated to zero time, and for valine and isoleucine, for which the 72 h values were used. The analyses did not include tryptophan.

		Composition (mol/mol)		
Amino acid	pI of transferase...	4.6	8.5	9.9
Asx		44.1	36.1	43.5
Thr		20.0	12.4	11.4
Ser		25.0	30.0	15.8
Glx		50.2	57.0	60.0
Pro		25.0	27.2	22.0
Gly		40.0	25.0	30.2
Ala		36.2	28.0	37.3
Val		23.1	16.0	31.4
Cys		8.0	10.0	14.4
Met		5.4	14.5	12.5
Ile		12.9	23.9	18.7
Leu		61.2	50.3	56.0
Tyr		22.6	18.0	19.1
Phe		16.4	17.0	19.0
Lys		28.0	48.0	38.7
His		4.5	6.2	8.6
Arg		15.7	17.8	26.7

Alpha transferases. The most basic skin transferase (at pH 9.9) gave a strong precipitin line with antibodies against rat transferase 2-2 and a weak line with antibodies against human Class Alpha transferases (Fig. 3). No other precipitin reactions were noted in any of the various combinations of antibodies and skin transferases possible.

Characterization of the basic human glutathione transferase of Class Alpha not previously described

The amino acid composition of the most basic glutathione transferase from human skin is clearly different from the compositions of the two additional major forms in the same tissue (Table 1). In spite of the structural differences, the transferases with pI values of 8.5 and 9.9 are similar in showing high specific activity with cumene hydroperoxide, a distinctive substrate for Class Alpha transferases (Mannervik *et al.*, 1985). The data presented in Table 2 show that the most basic transferase has the additional kinetic properties previously found to characterize Class Alpha transferases. The apparent subunit M_r (26500) of the most basic skin transferase is higher than the corresponding values for the skin transferase with pI 8.5 and the hepatic human enzymes first described (Kamisaka *et al.*, 1975). This difference in apparent M_r values is based on electrophoretic mobilities in SDS/polyacrylamide gels and is similar to the difference in mobilities noted previously for rat transferase subunits 1 and 2 (Jakoby *et al.*, 1984), which are subunits of enzymes that have also been referred to Class Alpha. Sequence analysis has recently demonstrated that the true M_r values of these rat enzyme

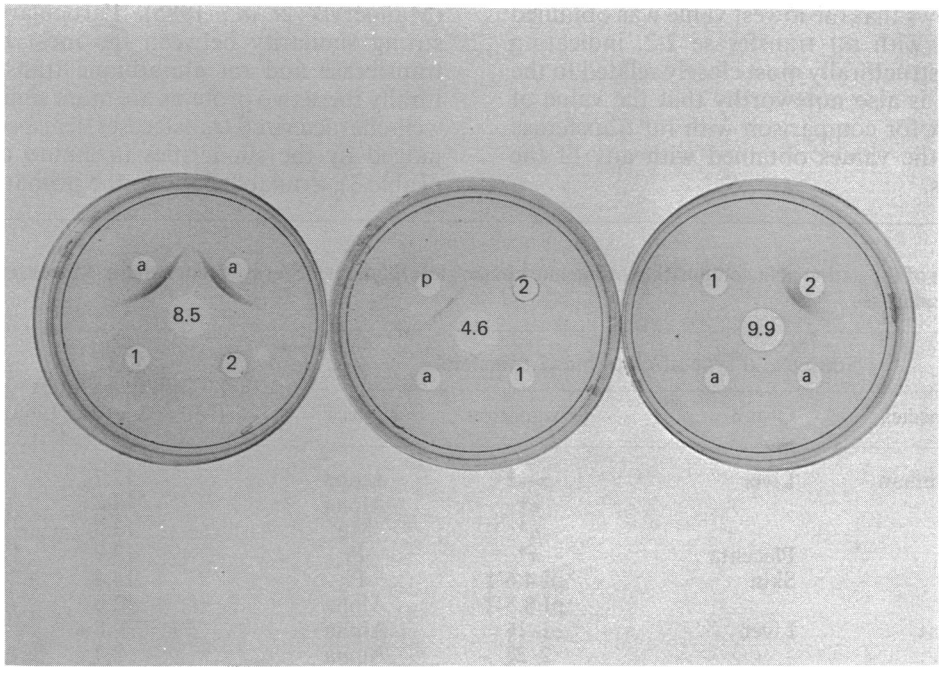

Fig. 3. Ouchterlony double-diffusion analysis of glutathione transferases isolated from human skin

The purified skin transferases (of the pI values shown) were placed in the centre wells, and antibodies raised against rat glutathione transferases 1-1 (1) and 2-2 (2) and human glutathione transferases α–ϵ (a) and π (p) were placed in the peripheral wells as indicated. The precipitin lines were stained with Coomassie Brilliant Blue R-250.

Table 2. Characterization of the most basic glutathione transferase from human skin by class-distinguishing substrates (*a*) and inhibitors (*b*)

(*a*) Substrate	Specific activity (μmol·min^{-1}·mg^{-1}) MBST*	TCAV†‡
Ethacrynic acid	0.31	0.01–1.2
Bromosulphophthalein	0§	< 0.01
trans-4-Phenylbut-3-en-2-one	0§	< 0.01
Cumene hydroperoxide	4.3	3–12
(*b*) Inhibitor	IC_{50} value (μM)‖ MBST*	TCAV†‡
Cibacron Blue	13	0.6–20
Triphenyltin chloride	1	0.3–30
Bromosulphophthalein	120	2–200
Haematin	1	0.05–2

* Most basic skin transferase, pI 9.9.
† Typical Class Alpha values.
‡ From Mannervik *et al.* (1985).
§ No detectable activity.
‖ IC_{50} is the inhibitor concentration giving 50% inhibition in the standard assay system.

subunits (Telakowski-Hopkins *et al.*, 1985), as well as of the rat enzyme subunit 4 (Ålin *et al.* 1986), are essentially the same.

In a search for structural similarities between the most basic human skin transferase and other glutathione transferases, the amino acid compositions were compared by use of the difference index described by Metzger *et al.* (1968). Table 3 shows that the lowest value was obtained in the comparison with rat transferase 2-2, indicating that this enzyme is structurally most closely related to the skin transferase. It is also noteworthy that the value of the difference index for comparison with rat transferase 1-1 is lower than the values obtained with any of the human transferases.

Table 4 shows the *N*-terminal amino acid sequence determined for the novel most basic glutathione transferase from human skin. For comparison, the corresponding structures of rat transferases 1-1 and 2-2 are given. The latter constitute the only representatives of the Class Alpha transferases previously determined. The *N*-terminal structure determined for the human transferase is identical with that of rat transferase 2-2 and differs in three positions from that of rat transferase 1-1. Comparisons with the known *N*-terminal amino acid sequences of transferases in Class Mu and Class Pi (Mannervik *et al.*, 1985) demonstrate that the latter enzymes are more distantly related (approx. 30% homology) to the most basic transferase from human skin discovered in the present investigation.

DISCUSSION

The results of this work show that human skin contains several glutathione transferases. The isoelectric-focusing profile in Fig. 1 is representative for the three samples from different individuals examined. The proportions and amounts of the different forms of glutathione transferase were essentially the same. A more careful analysis of the three major forms indicated that the most acidic of these (Fig. 1) is similar to, or identical with, human glutathione transferase π found in most tissues (Mannervik, 1985). The basic skin transferase with pI 8.5 appears similar to human transferases α–ϵ first isolated from liver (Kamisaka *et al.*, 1975).

The isolation and characterization of the most basic glutathione transferase in human skin represent the most significant part of the present investigation. This isoenzyme, with pI 9.9, which in skin accounts for 10–20% (on a protein basis) of the total amount of glutathione transferases, was previously unknown. By all the available criteria, it is a Class Alpha transferase (Mannervik *et al.*, 1985). Particularly striking is the strong similarity between the most basic human skin transferase and rat glutathione transferase 2-2. Structurally these two proteins are more similar than any other well-characterized transferases, irrespective of species, as judged by the similarities in amino acid compositions (Table 3), available data on the primary structure (Table

Table 3. Comparison of the amino acid composition of the most basic glutathione transferase from human skin with those of other human and rat transferases

Source and type of glutathione transferase				Difference index (Metzger *et al.*, 1968)
Species	Organ	Designation	Class	
Human	Liver	α–ϵ*	Alpha	12.7
		ϵ†	Alpha	14.0
		μ*	Mu	13.3
	Placenta	π*	Pi	13.9
	Skin	'pI 4.6'‡	Pi	14.4
		'pI 8.5'‡	Alpha	12.0
Rat	Liver	1–1§	Alpha	9.8
		2–2§	Alpha	5.4
		3–3§	Mu	15.4

* From Warholm *et al.* (1983).
† From Kamisaka *et al.* (1975).
‡ The present work (Table 1).
§ From Mannervik (1985); supplemented with the values for cysteine.

Table 4. *N*-terminal amino acid sequences determined for Class Alpha glutathione transferases

Position	Transferase ...	Amino acid: Most basic human skin*	Rat 2-2†	Rat 1-1‡
			(Met)	(Met)
1		Pro (130)	Pro	Ser
2		Gly (180)	Gly	Gly
3		Lys (240)	Lys	Lys
4		Pro (120)	Pro	Pro
5		Val (210)	Val	Val
6		Leu (280)	Leu	Leu
7		His (50)	His	His
8		Tyr (80)	Tyr	Tyr
9		Phe (170)	Phe	Phe
10		Asp (120)	Asp	Asn
11		Gly (90)	Gly	Ala
12		Arg (70)	Arg	Arg
13		Gly (40)	Gly	Gly
14		Arg (40)	Arg	Arg
15		Met (50)	Met	Met
16		Glu (30)	Glu	Glu

* The present work. Values within parentheses show pmol recovered in the analysis.
† Deduced from the cDNA structure (Telakowski-Hopkins *et al.*, 1985). Initiator methionine is indicated in parentheses.
‡ Deduced from the cDNA structure (Pickett *et al.*, 1984). Initiator methionine is indicated in parentheses. Differences from the other two sequences are boxed.

4) and reactions with specific antibodies (Fig. 2). The enzymic properties as expressed by specific activities and sensitivities to inhibitors are also similar (Table 2). High glutathione peroxidase activity with the organic hydroperoxides linolenic hydroperoxide (results not shown) and cumene hydroperoxide are particularly noteworthy.

Furthermore, the discovery of a human glutathione transferase of the Class Alpha type, for which an *N*-terminal amino acid sequence ascertains its kinship to the rat transferases, lends strong support to the recently proposed (Mannervik *et al.*, 1985) classification system.

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