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Identification of three classes of cytosolic glutathione transferase common to several mammalian species: Correlation between structural data and enzymatic properties

(isoenzymes/amino acid sequences/substrate specificities/inhibition characteristics/immunoprecipitation)

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ABSTRACT **The major isoenzymes of cytosolic glutathione transferase (EC 2.5.1.18) from rat, mouse, and man are shown to share structural and catalytic properties that can be used for species-independent classification. Rat, mouse, and human isoenzymes were grouped with respect to amino-terminal amino acid sequences, after correlation of seven structures analyzed in the present investigation with structures determined earlier. The isoenzymes were also characterized by substrate specificities and sensitivities to inhibitors, and the data were subjected to pattern recognition analysis. In addition, the various isoenzymes were tested for cross-reactivity by immunoprecipitation with antibodies raised against rat and human transferases. The different types of data were clearly correlated and afforded an unambiguous division of the isoenzymes into three classes named alpha, mu, and pi. Each of the three mammalian species studied contains at least one isoenzyme of each class. It is suggested that the similarities of the isoenzymes in a class reflect evolutionary relationships and that the classification applies generally.**

Glutathione transferases (EC 2.5.1.18) catalyze nucleophilic attack of glutathione on electrophilic centers in a wide variety of organic molecules (1, 2). The latter include xenobiotics as well as compounds endogenous to the organism. In particular, it has been emphasized that epoxides, organic hydroperoxides, and activated alkenals, resulting from oxidative metabolism, may be regarded as "natural" substrates for the glutathione transferases (3).

A prominent feature of glutathione transferase in an organism is the existence of isoenzymes. Virtually all species studied have been found to contain multiple forms of the enzyme (for a review, see ref. 4). For example, human tissues contain at least three classes of cytosolic glutathione transferase, distinguishable by physical, chemical, immunological, enzymatic, and structural properties (5–7). The differences suggest that these classes represent products of three different genes, in agreement with independent genetic studies (8, 9).

In spite of extensive knowledge of the properties of the multiple forms of both the human and the rat glutathione transferases, the possible relationships between different classes of the enzyme in one species to the classes in other species have remained obscure.

Table 1 summarizes the available information about the multiple forms of cytosolic glutathione transferase in rat, man, and mouse. The enzymes in all three species are dimers, and the subunits have similar sizes. The rat liver cytosol fraction contains in high concentration four subunits with distinct properties (10), now named 1, 2, 3, and 4 (11). Subunits 1 and 2 can form two homodimers and one heterodimer, giving rise to a "family" composed of the related transferases 1–1, 1–2, and 2–2. A similar family includes transferases 3–3, 3–4, and 4–4. No dimeric structures with combinations of subunits from the different families have been observed. Rat testis contains another major isoenzyme, transferase 6–6 (12, 13), and rat kidney expresses an additional form, transferase 7–7 (14). Glutathione transferases in man and mouse have not yet been named on the basis of subunit composition but are referred to by Greek letters (man) or other arbitrary designations (mouse).

Notwithstanding the difficulties in identifying classes of glutathione transferase in different species, it was recently noted (4) that the amino-terminal amino acid sequences of two homologous mouse transferases (15) showed extensive similarities to those of two homologous rat transferases (16). This finding prompted studies of the glutathione transferase isoenzymes in mouse liver (17) and in human tissues (7), as well as an attempt at interspecies classification of the multiple forms of the enzyme (18). The present report correlates enzymatic and structural distinctions and shows that glutathione transferases from several species can be assigned to three classes comprising isoenzymes with common characteristic properties.

MATERIALS AND METHODS

Enzymes. Glutathione transferases from human placenta (19), human liver (20), rat liver (21), rat kidney (14), rat testis (13), and mouse liver (17) were purified to homogeneity by procedures as indicated. Antibodies to purified transferases were raised in rabbits. Precipitin reactions were studied by the double-diffusion method of Ouchterlony.

Assay of Enzyme Activity. Activity measurements were made by standard procedures at 30°C (21, 22). The concentration of inhibitor giving 50% inhibition (IC_{50}) was determined as described (6).

Determination of Amino Acid Sequences. The proteins were reduced with dithiothreitol and carboxymethylated with iodo[^{14}C]acetate before liquid-phase sequencer degradations in Beckman 890D and modified (23, 24) 890C instruments. Phenylthiohydantoin derivatives were identified by their elution positions upon reverse-phase HPLC (25). [^{14}C]Carboxymethylcysteine derivatives were monitored also by liquid scintillation counting.

RESULTS

Structural Studies. Amino-terminal amino acid sequences of rat glutathione transferases 2–2, 3–3, 4–4, and 7–7 and of mouse glutathione transferases MII and MIII were determined and compared to the structures earlier analyzed (Table

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Table 1. Overview of the information available for the isoenzymes of glutathione transferase from rat, man, and mouse

Information	Rat	Man	Mouse
Well characterized isoenzymes	10	7	4
Nomenclature, subunit	Arabic numerals	Greek letters	Various
Structures analyzed			
Amino acid sequence data	3*	2	2†
cDNA sequence data	2	0	2
Enzymatic properties	+	+	+

*Increased to 5 by the results of this work.
†Increased to 4 by the results of this work.

2). In addition, results suggesting blocked amino-terminal amino acid residues were obtained for rat transferase 1–1 and mouse transferase MI. Evidence for blocked amino termini has been reported earlier for rat transferase 1–1 (32) and human basic transferases (7).

Table 2 shows that the sequences now available for isoenzymes of glutathione transferase can be segregated into three classes. Thus, mouse transferases MII and MIII, like the two human transferases π and μ analyzed (7), are discrete structures. The four rat isoenzymes have amino-terminal sequences of three different types; transferases 3–3 and 4–4 are clearly homologous. Following the previous grouping of human glutathione transferases (5), the structures have been arranged into three classes: alpha, mu, and pi.

The amino-terminal amino acid sequence determined for rat transferase 2–2 is identical to the corresponding structure reported for rat transferase B (now called 1–2) (16). Likewise, the amino-terminal sequences of rat transferases 3–3 and 4–4 agree with those reported for rat transferases A and X (16), respectively, confirming identity in each case. The subunits of mouse transferase MIII show corresponding extensive structural similarities with those of mouse transferases GT-8.7 and GT-9.3 (15). The bovine transferase analyzed (30) is assigned to class mu on the basis of its strict sequence homology with the other members of this class.

In addition to the protein structures determined, nucleotide sequences of cloned cDNA for rat subunits 1 and 2 have provided amino acid sequences (26–29) that are included in Table 2. Excluding the methionine residue, it appears significant that subunit 1 has serine as the amino-terminal amino acid, since no free amino group could be demonstrated and since serine residues are often *N*-acylated (33, 34).

Enzymatic Studies. Substrate specificities and inhibitor sensitivities that can be used to distinguish different isoenzymes are summarized in Table 3. The isoenzymes have been grouped under three headings (cf. Table 2). Class alpha is characterized by high activity with 1-methyl-1-phenylethyl hydroperoxide (cumene hydroperoxide); class mu by high activity with either *trans*-4-phenyl-3-buten-2-one or bromosulfophthalein (or 1,2-dichloro-4-nitrobenzene; data not shown); and class pi by high activity with ethacrynic acid, in comparison with all other substrates shown.

In terms of inhibition, class alpha is noted for a low IC_{50} value for hematin and a high value for Cibacron blue (the ratio of the IC_{50} values for a given isoenzyme being $\approx$0.1); class mu has a high IC_{50} value for hematin and a low value for Cibacron blue (the ratio of the IC_{50} values ranging from 3 to 20); class pi has a high IC_{50} value for hematin and a low value for Cibacron blue (the ratio being similar to class mu). The low IC_{50} values for triphenyltin chloride (0.04–0.5 μM) distinguish class mu from class pi, which has high values (>10 μM).

A more comprehensive analysis of the kinetic properties was made by multivariate analysis (35). The specific activities with 9 substrates and the IC_{50} values for 11 inhibitors were used as variables to characterize 15 isoenzymes of glutathione transferase. The set of variables defines for each enzyme a vector in 20-dimensional space. The analysis showed that the data could be adequately described by two principal components and that the representations of the isoenzymes in the plane spanned by these principal components were localized in three discrete domains (Fig. 1). Each of these domains contained all members of one of the three classes of glutathione transferase identified in this paper. In addition to the isoenzymes for which data are given in Tables 2 and 3, rat

Table 2. Amino-terminal amino acid sequences of glutathione transferases

Transferase	Amino acid sequence
Class alpha*	
Rat 1–1 (cDNA)	(M)S G K P V L H Y F N A R G R M E C I R W L L A A A
Rat 1–2	P G K P V L H Y F N G R G R M E P I
Rat 2–2	P G K P V L(H)Y F
Rat 2–2 (cDNA)	(M)P G K P V L H Y F D G R G R M E P I
Class mu†	
Human μ	P M I L G Y W D I R G L A H A I R L L L E Y T
Rat 3–3	P M I L G Y W N V R G L T H P I R L L
Rat 4–4	P M T L G Y W D I R G L A H A I R L F L E Y T D T
Mouse MIII	P M I L G Y W N V R G L T H P I R M L L Q Y T
Mouse GT-8.7	P M I L G Y X N V R G L X H P I R M L L E Y X D X
Mouse GT-9.3	P M T L G Y W N T R G L T H S I R L L L E Y X D S
Bovine enzyme	P M I L G Y W D I R G L A H A I S L L L
Class pi‡	
Human π	P P Y T V V Y F P V R G R C A A L R M L L A D
Rat 7–7	P P Y(T)I V Y F P V
Mouse MII	P P Y T I V Y F P V V D G C E A M

Residues within parentheses indicate tentative assignments or initiator methionine.
*Class alpha also comprises human transferases α–ε and mouse transferase MI. These proteins are amino-terminally blocked and are not listed owing to lack of sequence information. The structures derive from refs. 26–28 (rat 1–1, cDNA), ref. 16 (rat 1–2), ref. 29 (rat 2–2, cDNA), and present investigation (rat 2–2).
†The structures derive from ref. 7 (human μ), present work (rat 3–3 and 4–4, cf. ref. 16), present work (mouse MIII), ref. 15 (mouse GT-8.7 and GT-9.3), and ref. 30, (bovine).
‡The structures derive from refs. 7 and 31 (human π) and present work (rat 7–7 and mouse MII).

Table 3. Class-distinguishing substrate specificities and inhibitor sensitivities of glutathione transferases

Transferase class	Specific activity with substrate, μmol·min^{-1}·mg^{-1}					IC_{50} with inhibitor, μM			
	Ethacrynic acid	Bromosulfophthalein	*trans*-4-Phenyl-3-butene-2-one	Cumene hydroperoxide	Δ^5-Androstene-3,17-dione	Cibacron blue	Triphenyltin chloride	Bromosulfophthalein	Hematin
Alpha	0.01–1.2	<0.01	<0.01	**3–12**	**0.04–8**	0.6–20	0.3–30	**2–200**	**0.05–2**
Mu	0.08–0.6	**0.002–0.9**	**0.04–1.2**	0.1–0.7	0.002–0.1	**0.05–0.7**	**0.04–0.5**	0.5–10	1–2
Pi	**0.9–4**	<0.01	0.01–0.02	0.03–0.14	0.01–0.17	0.1–0.5	>10	20–100	4–5

Published data have been compiled from refs. 4–6, 10, 14, 17, 18, and 21. Characteristically high specific activities and low IC_{50} values are given in bold face. Members of class alpha or mu have high activity with at least one of the two substrates indicated. No characteristic inhibitor for class pi has yet been identified. Members of class pi are distinguished from those of classes alpha and mu by low sensitivity to both triphenyltin chloride and hematin.

transferase 6–6 as well as a rat transferase tentatively designated 3–? (21) were analyzed. Both of these enzyme forms appeared in the domain of class mu. Thus, pattern recognition analysis utilizing simultaneously the entire set of kinetic data for each isoenzyme leads to the same conclusion about classification of the multiple forms of glutathione transferase as the more limited direct comparison.

Studies with Specific Antisera. Polyclonal antisera raised against four rat transferases and three human transferases were tested against all the isoenzymes available from rat, mouse, and man. Table 4 shows the positive precipitin reactions in Ouchterlony double-diffusion assays. Most of the antisera reacted with at least one isoenzyme in addition to that against which they had been raised. Cross-reactivity among the three mammalian species was demonstrated. For example, anti-transferase α–ϵ antibodies precipitated human transferase α–ϵ and rat transferase 1–1, whereas anti-transferase π antibodies precipitated human transferase π, rat transferase 7–7, and mouse transferase MII. No cross-reactivity was noted between the classes, even within the same species. These results support the species-independent classification now proposed for the glutathione transferases and indicate that the structural similarities within a class extend beyond the amino-terminal regions.

DISCUSSION

Classification of Glutathione Transferases. Multiple forms of glutathione transferase have been demonstrated in many animal species (4). Analysis of the properties of the three major types of human glutathione transferase has provided evidence for the existence of three distinct types of the enzyme (5–7). These types have been referred to as basic (or α–ϵ), near-neutral (or μ), and acidic (or π) (36). The present investigation demonstrates that the classification used for the human enzymes can be extended to cytosolic glutathione transferases from other mammalian species and that each of the isoenzymes of glutathione transferase studied here can be uniquely assigned to one class. Strong support for the classification comes from the correlation of amino acid sequences with the kinetic properties and the immunochemical reactions used for discrimination. In the case of the enzymes referred to class alpha, all polypeptide chains except rat subunit 2 appear to have blocked amino termini, at this stage preventing structural comparisons of amino-terminal amino acid sequences. Nevertheless, the data presented in Tables 2–4 and Fig. 1 suggest strongly that these isoenzymes are closely related.

In addition to the properties discussed above, it may be added that the apparent subunit M_r values derived from sodium dodecyl sulfate/polyacrylamide gel electrophoresis suggest differences between the three classes. In each of the species studied, it was found that subunits of class pi have the lowest apparent M_r, class alpha an intermediate M_r, and class mu the highest M_r. The exception is rat subunit 2 (of class alpha) which appears to have the highest M_r. It was recently found that rat subunits 1 and 2, which differ in M_r by 2000, judging from their electrophoretic mobilities, have virtually identical M_r values (25,500) as deduced from cDNA sequences (29). The M_r value for subunit 3 was similarly reported as 25,900. Irrespective of the explanation for these

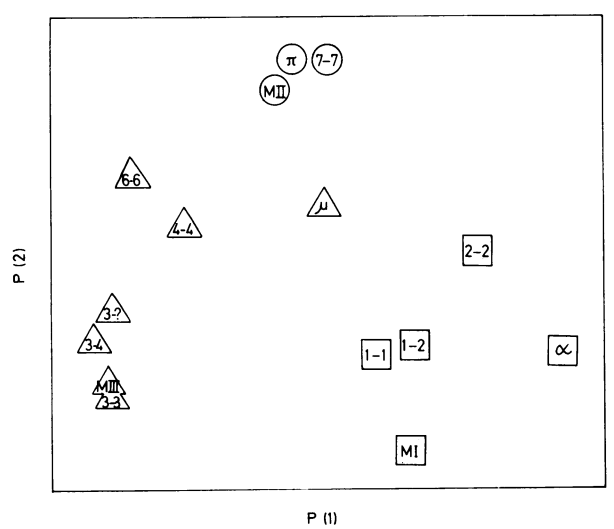

FIG. 1. Pattern recognition analysis of enzymatic properties of glutathione transferase isoenzymes from rat, mouse, and man. The first, $P(1)$, and second, $P(2)$, principal components computed for each isoenzyme by multivariate analysis (35) on the basis of specific activities with 9 substrates and IC_{50} values for 11 inhibitors are plotted. The symbols were chosen to designate class alpha (□), class mu (△), and class pi (○).

Table 4. Reaction of polyclonal antibodies with purified glutathione transferases

Transferase	Reaction with antibodies to transferase						
	Rat 1–1	Rat 2–2	Human α–ϵ	Rat 3–3	Rat 4–4	Human μ	Human π
Class alpha							
Rat 1–1	+		+				
Rat 2–2		+					
Human α–ϵ	+		+				
Mouse MI		+					
Class mu							
Rat 3–3				+			
Rat 4–4				+	+		
Human μ						+	
Mouse MIII				+	+		
Class pi							
Rat 7–7							+
Human π							+
Mouse MII							+

+, presence of a precipitin line in an Ouchterlony double-diffusion immunoassay.

results, the relative electrophoretic mobilities give further support to the classification.

On the other hand, the isoelectric points of the different isoenzymes cannot be used for a general classification into groups of basic, near-neutral, or acidic transferases in the way used for the human enzymes (36). For example, all of the major mouse and most of the rat transferases are basic proteins. Even the relative orders of the isoelectric points of the transferases change from species to species when the classes are compared.

The sequences in Table 2 show similarities within a class as well as between classes. However, it is evident that the differences between the three classes of glutathione transferase in a single mammalian species are greater than the differences between the enzymes from different species within a class. Thus, the classes probably diverged before the evolution of different mammalian species. Fig. 2 summarizes the classification of the various isoenzymes of cytosolic glutathione transferase and shows their suggested relationships. The existence of a microsomal glutathione transferase (37) is also indicated. By reference to amino-terminal amino acid sequences, enzymatic properties, or immunochemical reactions, it should be possible to relate additional isoenzymes to the classes defined here.

Nomenclature. Glutathione transferases were originally named on the basis of assumed specificities for functional groups of electrophilic substrates, such as aryl, alkyl, alkene, and epoxide groups (38). This principle was abandoned as soon as it had been demonstrated that isolated transferases had overlapping substrate specificities. The enzymes were then denoted by Roman letters (for rat transferases) and Greek letters (for human transferases) (39). It was later suggested that the names of the isoenzymes should reflect their respective subunit composition (10), and the subunits of the well-characterized glutathione transferases from rat cytosol fractions were recently named by Arabic numerals (11). Similar designations of human and mouse transferases have been considered.

However, none of the nomenclature systems for the glutathione transferases gives any clues to the relatedness between subunits. For example, the strict amino acid sequence homology between rat subunits 3 and 4 is not reflected in their designations. Nor is their homology with human transferase μ indicated in any way. Therefore, a unifying classification system such as the one put forward in this report is needed. By noting that transferase MIII from mouse liver belongs to class mu, structural homology and functional similarities with the other members of the class are immediately indicated. Similar relationships become obvious between the isoenzymes contained in the other classes. It would appear that this classification also reflects discrete biochemical functions for the enzymes in the three classes. For example, the members of class alpha display high glutathione peroxidase activity. In choosing the designations for the three classes of glutathione transferase, the names of Greek letters for representative human enzymes were used. Other systems based on Arabic or Roman numerals or Roman letters were considered unsuitable owing to the risk of confusion of individual enzymes with classes to which they do not belong.

Finally, it should be noted that additional classes of glutathione transferase might exist even though the classification made here includes nearly all of the individual cytosolic isoenzymes that have been carefully characterized in rat, mouse, and human tissues. An additional class might contain the microsomal glutathione transferase isolated from rat liver, which is clearly distinct from the cytosolic isoenzymes (37). Its amino acid sequence has been determined (40), and it appears to lack obvious homology with any of the cytosolic enzymes presented in Table 2.

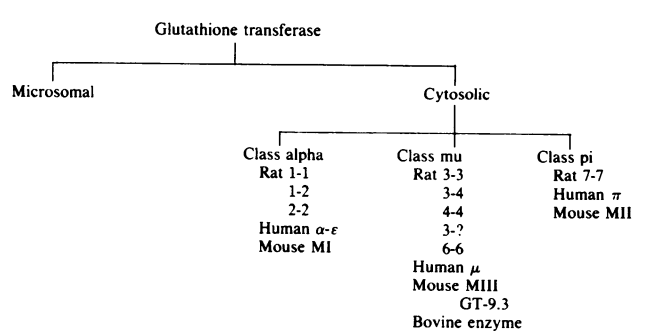

FIG. 2. Classification and relationships of glutathione transferases. For synonymous designations of individual isoenzymes see refs. 4 and 11.

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