Glutathione transferases in primary rat hepatomas: the isolation of a form with GSH peroxidase activity

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Received 8 March 1985

A previously uncharacterized glutathione (GSH) transferase which is not apparent in normal liver, accounts for at least 25% of the soluble GSH transferase content of primary hepatomas induced by feeding N,N-dimethyl-4-aminoazobenzene. This enzyme is readily isolated, has an isoelectric point of 6.8, is composed of two identical subunits of apparent M_r 26000 and has GSH transferase activity towards a number of substrates including benzo(a)pyrene-7,8-diol-9,10-oxide. It is unusual in that it has GSH peroxidase activity towards fatty acid hydroperoxides but not towards the model substrates, cumene hydroperoxide and *t*-butyl hydroperoxide. It has been shown by tryptic peptide analysis to be distinct from GSH transferases composed of subunits 1, 2, 3, 4 or 6 and has been designated GSH transferase 7-7.

GSH transferase 7-7 *GSH peroxidase* *Hepatoma* *Purification*

1. INTRODUCTION

An early macroscopic event in experimental chemical hepatocarcinogenesis is the formation of hyperplastic nodules and although most of these nodules subsequently revert to apparently normal hepatic tissue some persist and proceed to hepatocarcinomas. Farber and his colleagues have shown that a variety of carcinogenic regimens give rise to nodules with similar phenotypic characteristics many of which they have pointed out are suggestive of adaptation to a toxic environment (reviewed in [1]). For example, marked decreases in the enzymes which produce electrophiles such as mixed function oxygenases are combined with increases in factors involved in the detoxication of electrophiles and the excretion of xenobiotics such as GSH, GSH transferases, γ-glutamyl transpeptidase, NAD(P)H-quinone reductase, microsomal epoxide hydrolase and UDP-glucuronyl transferase [2].

The glutathione (GSH) transferases are our particular interest. They are a family of dimeric isoenzymes existing as homodimers and heterodimers with a distribution which is specific to a tissue and perhaps relevant to its function [3]. Here, we have examined a series of hepatomas brought about by feeding *N,N*-dimethyl-4-aminoazobenzene (DAB) and isolated and characterized a GSH transferase abundant in all these hepatomas, present in several normal tissues examined, but not evident in normal liver. It has been agreed by the consulting group for the nomenclature of GSH transferases [4] it should be called GSH transferase 7-7.

2. MATERIALS AND METHODS

2.1. *Induction of hepatomas by* N,N-*dimethyl-4-aminoazobenzene (DAB)*

Tumour-bearing rats were kindly provided by Dr J. Embleton, Cancer Research Campaign Laboratories, University of Nottingham. They were male Wistar rats, initially weighing 100 g, which were fed a powdered diet containing 0.06% DAB, 0.2% corn oil, 9.8% dried carrot and 88.2% unpolished rice for 3 months, then transferred to a standard laboratory diet until hepatomas were judged to be present (in the region of 8 months).

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00145793/85/$3.30

The rats were then killed, hepatomas were dissected free of surrounding tissue, frozen in liquid nitrogen and stored at −70°C prior to use.

2.2. *Enzyme assays*

GSH transferase, GSH peroxidase and Δ^5-3-keto-steroid isomerase activities towards model substrates were determined according to [5]. GSH transferase activity towards the carcinogens *N*- sulphonyloxy-*N*-acetyl-2-aminofluorene, *N*-acetoxy-*N*-acetyl-2-aminofluorene (prepared according to [6]) and benzo(*a*)pyrene-7,8-diol-9,10-oxide (supplied by Dr Bengt Jernström, Karolinska Institutet, Stockholm) was determined by measuring respective GSH conjugates separated by HPLC [7]. Hydroperoxides of the naturally occurring linoleic and arachidonic acids prepared as described by O'Brien [8] were used as GSH peroxidase substrates.

2.3. *Separation of GSH transferases*

Hepatoma tissues were thawed, minced and homogenised in two volumes of buffer containing 0.2 M sucrose, 2 mM EDTA, 0.5 mM PMSF, 10 mM KH_2PO_4 and 15 mM Tris base adjusted to pH 7.8 with KOH and the soluble supernatant fraction obtained by centrifugation at 105000 × *g* for 1 h. The GSH transferase fraction was separated by use of the affinity matrix *S*-hexylglutathione-Sepharose and from it GSH transferase 7-7 was purified by adsorption onto a column of hydroxyapatite (1 × 20 cm) in 7 mM sodium phosphate, 1 mM EDTA (pH 6.5) and elution with a gradient of potassium phosphate. Fractions containing GSH transferase 7-7 were eluted at approx. 20 mM potassium phosphate, and were pooled and re-chromatographed on hydroxyapatite to yield a homogenous protein. Glutathione transferase 7-7 was isolated in a similar fashion from kidney, skeletal muscle, testis and epididymis. The purity of the GSH transferase 7-7 preparation was assessed by SDS-PAGE (polyacrylamide gel electrophoresis) and isoelectric focusing on a sucrose density gradient.

A comparison of the soluble GSH transferases obtained in hepatoma with those in normal liver was made by subjecting eluates from *S*-hexylglutathione-Sepharose affinity columns to isoelectric focusing.

2.4. *Protein analysis*

The amino acid content of GSH transferase 7-7 was determined by Dr A. Aitken, School of Pharmacy, University of London and tryptic peptide maps were prepared as previously described [5].

2.5. *Immunology*

Antisera to GSH transferase 7-7 were raised in mice and tested by standard radioimmunoassay procedures using ^{125}I-labelled sheep anti-mouse $F(Ab^1)_2$ provided by Dr D. Male, Dept. of Immunology, Middlesex Hospital Medical School, London.

3. RESULTS

3.1. *Purification and characterisation of GSH transferase 7-7*

GSH transferase 7-7 was readily purified by chromatography on hydroxyapatite since it was desorbed at much lower phosphate concentration than other GSH transferases (fig.1). It gave a major peak at p*I* 6.8 on isoelectric focusing and a single band on SDS-PAGE migrating faster than subunit 1 and with an apparent M_r of 26000. Its amino acid content shown in table 1 is unusual among GSH transferase so far studied for its very

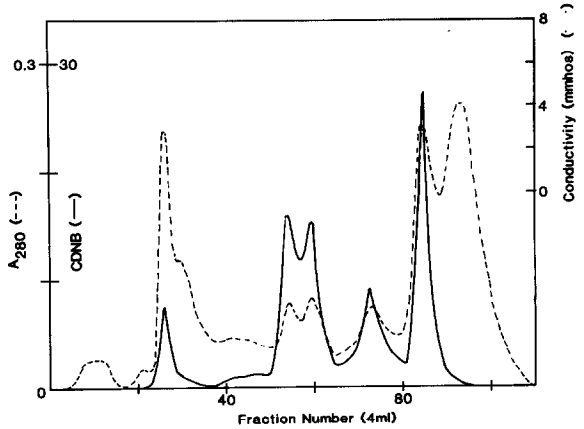

Fig.1. Purification of GSH transferase 7-7. The GSH transferase fraction obtained by affinity chromatography from a 15 g DAB-induced hepatoma was applied to a column of hydroxyapatite as described in section 2. GSH transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) is given in μmol/min per ml. Fractions 25–28 were re-chromatographed to yield an apparently pure enzyme.

Table 1

The amino acid composition of GSH transferases 1-1, 2-2, 3-3, 4-4 and 7-7

Amino acid	GSH transferase[a]				
	1-1	2-2	3-3	4-4	7-7
Asx	10.1	10.3	11.1	11.0	12.3
Thr	3.5	3.2	3.0	3.5	3.5
Ser	4.4	4.1	4.6	5.7	4.9
Glx	11.7	11.7	11.6	11.5	10.9
Pro	4.9	4.5	6.0	6.4	4.8
Gly	5.4	5.6	4.8	5.9	9.6
Ala	7.5	7.6	5.2	5.8	6.8
Val	4.7	4.9	2.2	3.1	6.2
Cys	0.9	0.9	1.1	0.9	2.1
Met	3.8	3.6	3.6	2.8	<0.3
Ile	4.5	5.0	5.5	4.3	3.6
Leu	13.0	12.6	11.5	10.7	14.1
Tyr	3.5	3.7	6.6	5.6	3.6
Phe	4.5	4.2	5.8	6.2	3.1
His	1.4	1.5	1.7	2.3	3.2
Lys	9.6	9.9	9.1	8.4	6.2
Arg	6.1	6.3	6.2	5.4	3.4
Trp	0.5	0.5	0.5	0.5	1.4

[a] Amino acid content is expressed as mol%

low level of methionine and relatively high level of glycine. The expected yield of tryptic peptides from subunits of a homodimer with this composition is 26. 31 peptides are found in the tryptic peptide map shown in fig.2. This value is in sufficient agreement with the amino acid analysis to indicate that the subunits in GSH transferase 7-7 are identical or, as with GSH transferase 1-1, closely homologous.

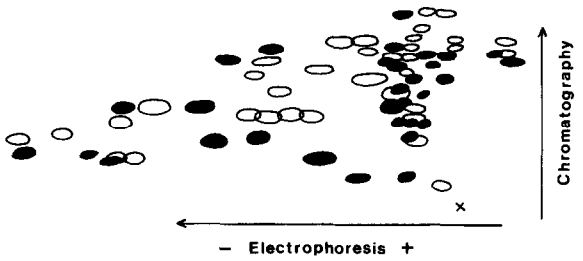

Fig.2. Tryptic peptides of GSH transferase 7-7. Purified GSH transferase 7-7 was compared in this example with GSH transferase 3-3 by thin layer tryptic peptide mapping as described in [5].

Comparison of the tryptic peptide map for GSH transferase 7-7 with those of GSH transferases 1-1, 2-2, 3-3 (see fig.2), 4-4 or 6-6 suggested much less homology between subunit 7 and subunits 1, 2, 3, 4 or 6 than has been shown to exist [5] between subunits 1 and 2 on the one hand and subunits 3, 4 and 6 on the other. Also antibody raised to GSH transferase 7-7 gave very low titres with GSH transferases 1-2 and 3-4.

3.2. *Enzymic properties and tissue distribution of GSH transferase 7-7*

Enzymic activities of GSH transferase 7-7 are shown in table 2. It has one characteristic which is unique so far, namely while it has GSH peroxidase activity towards fatty acid hydroperoxide it is almost inactive with the usual model organic substrates for GSH peroxidase activity, cumene hydroperoxide and *t*-butyl hydroperoxide. This is its main distinguishing characteristic, but high activity towards ethacrynic acid is also noteworthy. Otherwise it utilizes 1-chloro-2,4-dinitrobenzene as a substrate like most other GSH transferases but is inactive towards Δ^5-androstene-3,17-dione, cumene hydroperoxide, 1,2-dichloro-4-nitrobenzene and *trans*-4-phenyl-3-buten-2-one which are substrates used to identify subunits 1, 2, 3, 4 respectively. It has appreciable activity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane but substantially less than that of GSH transferase 5-5. Highly reactive carcinogen metabolites have also been tested. Like all GSH transferases tested so far it is active towards benzo(*a*)pyrene-7,8-diol-9,10-oxide [7,9] and inactive towards *N*-sulphonyloxy-*N*-acetyl-2-aminofluorene or its analogue *N*-acetoxy-*N*-acetyl-2-aminofluorene [9].

Among tissues which were examined, namely primary hepatoma, normal liver, kidney, skeletal muscle, testis and epididymis, GSH transferase 7-7 was a major component of hepatoma, kidney and skeletal muscle, present in testis and epididymis, but not evident in liver.

In the hepatoma GSH transferase 7-7 accounted for about 25% of the total GSH transferase protein and the content of GSH transferase 3-3 was almost twice its level in normal liver (fig.3). The levels of GSH transferases 1-1, 1-2 and 2-2 were reduced to varying extents in the hepatomas compared to normal liver. Other isoenzymes were at similar levels in both tissues. Similar patterns of

Table 2

Activity of GSH transferase homodimers to a range of substrates

	1-1	2-2	3-3	4-4	5-5	6-6	7-7
1-Chloro-2,4-dinitrobenzene	34	32	44	16	tr.	187	20
1,2-Dichloro-4-nitrobenzene	0.15	0.15	8.4	0.7	nil	0.26	nil
trans-4-Phenyl-3-buten-2-one	0.1	0.1	0.1	1.2	nil	0.1	0.02
p-Nitro-phenethyl bromide	nd	nd	0.1	nd	4.2	nd	nil
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	0.7	0.9	0.2	0.9	25.5	0.1	1.0
Ethacrynic acid	0.3	2.1	0.4	1.0	nil	0.1	4.0
Cholesterol α-epoxide[a]	0.14	0.14	nil	nil	nil	nd	nd
Benzo(*a*)pyrene-7,8-diol-9,10-epoxide	0.10	0.08	0.03	0.36	nil	0.19	0.33
N-Sulphonyloxy-*N*-acetyl-2-aminofluorene	nil	nil	nil	nil	nil	nil	nil
Cumene hydroperoxides	1.4	3.0	0.1	0.4	12.5	0.04	0.01
t-Butyl hydroperoxide	0.1	0.5	0.04	0.1	nd	nd	0.02
Linoleate hydroperoxides	3.0	1.6	0.2	0.2	nd	0.06	1.5
Arachidonate hydroperoxides	2.6	1.7	0.2	0.2	nd	nd	1.5
Δ^5-Androstene-3,17-dione	0.23	0.07	nd	nd	nd	nd	<0.001

Activities are expressed as μmol/min per mg protein or [a] nmol/min per mg protein. nd, not determined; tr., trace

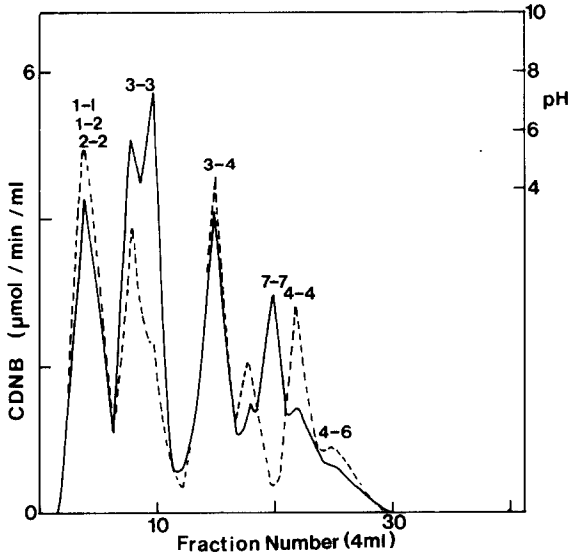

Fig.3. Comparison of the GSH transferase content of primary hepatoma with that of normal liver. The GSH transferase fractions were purified by affinity chromatography, subjected to isoelectric focusing in a sucrose gradient [5] and identified by assaying activity with a range of substrates combined with electrophoretic analysis of subunit composition as described in section 2. GSH transferase activity from a 3.5 g hepatoma (——) is compared with that from 3.5 g of normal liver (– – –).

GSH transferase were obtained with primary hepatomas ranging in weight from 0.5 to 20 g.

4. DISCUSSION

This paper describes a GSH transferase which accounts for 25% of the total GSH transferase of hepatoma and is also present in kidney, skeletal muscle, testis and epididymis, kidney being the richest source of this enzyme so far among normal tissues. Mannervik et al. (personal communication) have also isolated an enzyme from kidney which appears to be identical to the enzyme described here. By reference to Jakoby et al. [4] the enzyme has been named GSH transferase 7-7. This enzyme is probably identical to a GSH transferase isolated from hyperplastic hepatic nodules, placenta, kidney and lung by Sato et al. [10,11] and referred to as GST (P). It is also most probably identical to the marker protein for preneoplasia, p21, first seen by Eriksson et al. [12] and the p26-6.8 protein seen in both preneoplastic nodules and primary hepatoma by Sugioka et al. [13].

GSH transferase 7-7 utilizes a wide range of substrates including the ultimate carcinogen benzo(*a*)pyrene-7,8-diol-oxide. It is unique so far in having GSH peroxidase activity for the biologically significant arachidonic and linoleic acid hydro-

peroxides but not the model substrates cumene hydroperoxide and *t*-butyl hydroperoxide.

It is particularly interesting in that like other GSH transferases it does not catalyse the GSH conjugation of the electrophilic metabolite of *N*-acetyl-2-aminofluorene (AAF), *N*-sulphonyloxy-AAF, nor the synthetic analogue *N*-acetoxy-AAF. Thus, the presence of GSH transferase 7-7 is unlikely to contribute to the resistance to AAF toxicity which characterises hyperplastic nodules [1].

ACKNOWLEDGEMENT

We are grateful to the Cancer Research Campaign, of which B.K. is a Fellow, for generous support.

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