Biochemical Pharmacology, Vol. 56, pp. 763–771, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S006-2952(98)00065-3

Hepatic Mercapturic Acid Formation: Involvement of Cytosolic Cysteinylglycine S-Conjugate Dipeptidase Activity

*Claudio Jösch, Helmut Sies and Theodorus P. M. Akerboom**

INSTITUT FÜR PHYSIOLOGISCHE CHEMIE I, HEINRICH-HEINE-UNIVERSITÄT, DÜSSELDORF, POSTFACH 101007, 40001 DÜSSELDORF, GERMANY

ABSTRACT. The role of cysteinylglycine S-conjugate dipeptidases in the intrahepatic mercapturic acid pathway was investigated in rat liver. Subcellular compartmentation studies and liver perfusions were performed using monochlorobimane and bimane S-conjugates as model compounds. The major part (over 95%) of total hepatic cysteinylglycine S-conjugate dipeptidase activity was located in the cytosol. Lower specific activity appeared in the canalicular plasma membrane fraction. Similar hepatic localization of dipeptidase activity was seen in the guinea pig. In intact rat liver perfused with monochlorobimane, the major products were the glutathione S-conjugate (mBSG) and the cysteinylglycine S-conjugate (mBCG) in bile. Minor amounts of the cysteine S-conjugate (mBCys) and the mercapturic acid (mBNAc) were formed, indicating a limitation in further metabolism of the dipeptide S-conjugate in the biliary space. However, when the dipeptide S-conjugate was offered to the sinusoidal space in liver perfusions, substantial uptake and conversion to mBNAc was observed, and only trace amounts of the infused dipeptide appeared in bile. The data suggest that cytosolic cysteinylglycine S-conjugate dipeptidase as identified here is involved in hepatic mercapturic acid formation from sinusoidal cysteinylglycine S-conjugates. This is especially of significance for species such as guinea pig and human, in which dipeptide S-conjugates are generated in the sinusoidal domain of the liver due to the presence of high γ-glutamyltranspeptidase activity. BIOCHEM PHARMACOL **56**;6:763–771, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cysteinylglycine S-conjugate; dipeptidase; glutathione; mercapturic acid; monochlorobimane; rat liver perfusion

The liver plays a major role in the glutathione transferase-catalyzed formation of glutathione S-conjugates as the initial step of the mercapturic acid pathway, involved in detoxication and excretion of xenobiotics [1–4]. In the rat, glutathione S-conjugates formed in the liver are excreted into bile, and to a lesser extent into the systemic blood [5, 6]. An interorgan metabolism has been described in which systemic glutathione S-conjugates are subject to renal degradation via membrane-bound γ-GT† and dipeptidases, followed by *N*-acetylation to mercapturic acid via microsomal *N*-acetyl-transferase in the liver [7]. The metabolism of biliary glutathione S-conjugates may involve an enterohepatic process [8]. In mammalians such as guinea pig and human, high in hepatic γ-GT activity, an intrahepatic mercapturic acid pathway has been reported to be of importance [9–11].

Two membrane-bound dipeptidases, APM (EC 3.4.11.2) and DHP-I (EC 3.4.13.11) are thought to be responsible for the hydrolysis of cysteinylglycine S-conjugates [12–15]. The enzymes differ concerning their tissue distribution. In the rat, APM activity is highest in kidney, brain and small intestine, while DHP-I activity is high in lung and kidney, but not in liver [16].

The aim of the present work was to explore the hepatic subcellular distribution of cysteinylglycine S-conjugate dipeptidase activities and its possible relevance for intrahepatic mercapturic acid formation in guinea pig and rat. Monochlorobimane and the mercapturic acid pathway intermediates derived therefrom were used as a homolog series of substrates to study the different enzyme activities. Metabolic rates in the intact organ were assessed in *in situ* rat liver perfusion studies.

MATERIALS AND METHODS

Materials

mBCl and mBBr were purchased from Molecular Probes Europe BV (Leiden). DMSO was obtained from EGA-

* Corresponding author: Dr. T. Akerboom, Institut für Physiologische Chemie I, Heinrich-Heine-Universität Düsseldorf, Postfach 101007, 40001 Düsseldorf, Germany. Tel. +49–211-8112723; FAX +49–211-8113029; E-mail: theodorus.akerboom@uni-duesseldorf.de.

† *Abbreviations:* APM, aminopeptidase M; DHP-I, dehydropeptidase I; γ-GT, γ-glutamyltranspeptidase; GST, glutathione transferase; mBBr, monobromobimane; mBCG, bimane S-cysteinylglycine; mBCl, monochlorobimane; mBCys, bimane S-cysteine; mBNAc, bimane S-(*N*-acetyl) cysteine; mBSG, bimane S-glutathione; and TCA, trichloroacetic acid

Received 11 September 1997; accepted 20 January 1998.

Chemie, *N*-acetylcysteine was from Fluka, and other chemicals and biochemicals from Merck, Sigma-Aldrich or Boehringer–Mannheim. SepPak-C_{18} cartridges were obtained from Millipore Corp.

Synthesis of Bimane S-Conjugates

The bimane S-conjugates of glutathione, cysteinylglycine, cysteine, and *N*-acetylcysteine were synthesized by reaction of mBBr with the respective thiols by a procedure modified from [17] as follows. Three milligrams of mBBr were dissolved in 3 mL of ethanol, and 1.5 equivalents of the thiol dissolved in 3 mL of 100 mM sodium phosphate (pH 8) were added and incubated under nitrogen in the dark for 2 hr. The mixture was evaporated at 40° to 1 mL of final volume and extracted with 5 mL of dichloromethane three times to remove unreacted mBBr. Traces of dichloromethane were removed under reduced pressure and the solution was brought to pH 3 with 1 M HCl. Further purification was performed via elution through SepPak C_{18} pretreated with 2 mL of methanol and 5 mL of distilled water. After loading the sample, the SepPak cartridge was subsequently washed with 4 mL of 100 mM sodium phosphate buffer (pH 8) to remove the thiols and thiol disulfides, with 3 mL of water, which removes the salts, and with 4 mL of a 1:1 methanol/water mixture to elute the product. The eluate was dried under a stream of nitrogen and the final product (~5 mg) dissolved in 1 mL of water and stored at −20°. The purity was checked by TLC on cellulose F_{254} plates (Merck) using a mixture of 1-butanol/acetic acid/pyridine/water (15:3:10:12 v/v) as the mobile phase and detection by fluorescence, UV absorbance and ninhydrin staining. Complete removal of the starting thiols was examined by the method of Ellman [18]. Concentrations were determined by absorption measurements at 390 nm using the molar extinction coefficient of 5300 for mBSG [19], found to be valid for the other S-conjugates as well (data not shown).

HPLC Analysis

An HPLC system with fluorescence detector from Merck-Hitachi was used for quantitative analysis of the bimane S-conjugates. Elutions were performed isocratically on a LiChrospher reversed phase C_{18} endcapped column (RP_{18}e, 250 × 4 mm, 5 μm) including a LiChrospher precolumn (RP_{18}e, 4 × 4 mm) with a mobile phase consisting of 8% methanol in 20 mM sodium acetate, pH 3.5 at a flow rate of 1 mL × min^{-1}. Fluorometric detection was performed at an excitation wavelength of 390 nm and an emission wavelength of 482 nm. Retention times for mBSG, mBCG, mBCys, and mBNAc were 20, 12, 10, and 46 min, respectively.

Rat and Guinea Pig Liver Fractionation

Homogenate, cytosolic and microsomal fractions from livers of male Wistar rats and male guinea pigs, fed *ad lib.* and weighing 250–300 g, were prepared as described in [20]. Basolateral and canalicular enriched liver plasma membranes of both species were isolated according to the method as described in [21].

Rat Liver Perfusions

Rat livers were perfused *in situ* in a (non-recirculating) single pass mode with Krebs–Henseleit buffer saturated with carbogen (95% O_2/5% CO_2) at pH 7.4 and 37° with an influent flow rate of 3–4 mL × min^{-1} × g of $liver^{-1}$ with the bile duct cannulated as described previously [22]. Additions were made from stock solutions via infusion pumps shunted shortly before the liver [23]. mBCl was dissolved in DMSO and the bimane S-conjugates in distilled water. Effluent samples were deproteinized by adding 150 μL of TCA (50%) to 1350 μL of sample. Bile was collected during 5-min periods in Eppendorf tubes containing 50 μL of 10% TCA, centrifuged, and the supernatant diluted 20-fold with 5% TCA. The acidified perfusate and bile samples were extracted with 500 μL of dichloromethane in order to remove lipids prior to HPLC analysis.

Enzyme Assays

Marker enzymes were assayed spectrophotometrically, lactate dehydrogenase as described in [24], NADPH-cytochrome c oxidoreductase as in [25], Na,K-ATPase as in [26], APM as in [27], and γ-GT as in [28] with γ-glutamyl-3-carboxy-*p*-nitroanilide as the artificial substrate.

For measurement of the mercapturic acid pathway enzyme activities, methods described for glutathione transferase [29], γ-GT [28], DHP-I [16], and N-acetyltransferase [7] were modified by replacing the substrates for 1 mM mBCl or the corresponding bimane S-conjugates. Bestatin (2 mM) was added in the γ-GT assay to prevent further metabolism of the reaction product via dipeptidases. Incubations were performed for 10 min at 37° at a volume of 1 mL, and 150 μL of aliquots were taken at 2-min time intervals and stopped with 1350 μL of 5% TCA. Lipids and nonreacted mBCl in the GST assay were removed from the samples by extraction with 500 μL of dichloromethane prior to analysis of the reaction products by HPLC. Protein was assayed according to Bensadoun and Weinstein [30].

RESULTS

Mercapturic Acid Pathway Enzyme Activities Measured with Bimane S-Conjugates in Rat and Guinea Pig Livers

Activities of the mercapturic acid pathway enzymes were measured in homogenates of rat and guinea pig liver (Table 1) using monochlorobimane and the respective bimane S-conjugates as substrates (Fig. 1). In rat liver, GST exhibited by far the highest activity (310 nmol × min^{-1} × mg of $protein^{-1}$), demonstrating the high capacity of this organ for glutathione S-conjugate formation. Cysteinylgly-

TABLE 1. Specific activities of mercapturic acid pathway enzymes in rat and guinea-pig liver homogenates

Species	Enzyme			
	GST	γ-GT	mBCG-dipeptidase	*N*-acetyltransferase
Rat	308 ± 24	0.73 ± 0.04	6.1 ± 0.9	0.56 ± 0.15
Guinea pig	193 ± 14	3.5 ± 0.2	2.7 ± 0.8	0.61 ± 0.1

mBCl, mBSG, mBCG, and mBCys were used as the respective substrates as described in Materials and Methods. Values are means ± SEM (N = 3–4). Enzyme activities are expressed in nmol × min^{-1} × mg of $protein^{-1}$.

cine S-conjugate dipeptidase activity was also found to be relatively high (6.1 nmol × min^{-1} × mg of $protein^{-1}$) as compared to γ-GT (0.73 nmol × min^{-1} × mg of $protein^{-1}$) and *N*-acetyltransferase (0.56 nmol × min^{-1} × mg of $protein^{-1}$). A similar activity pattern was observed in guinea pig liver, except for γ-GT, which was *ca.* five times higher than in the rat.

Subcellular Distribution of γ-GT and Cysteinylglycine S-Conjugate Dipeptidase Activities in Rat Liver

While glutathione S-conjugate formation and *N*-acetylation are intracellular events, γ-glutamyltranspeptidation and dipeptide S-conjugate hydrolysis are thought to occur via plasma membrane-bound ectoenzymes. In accordance

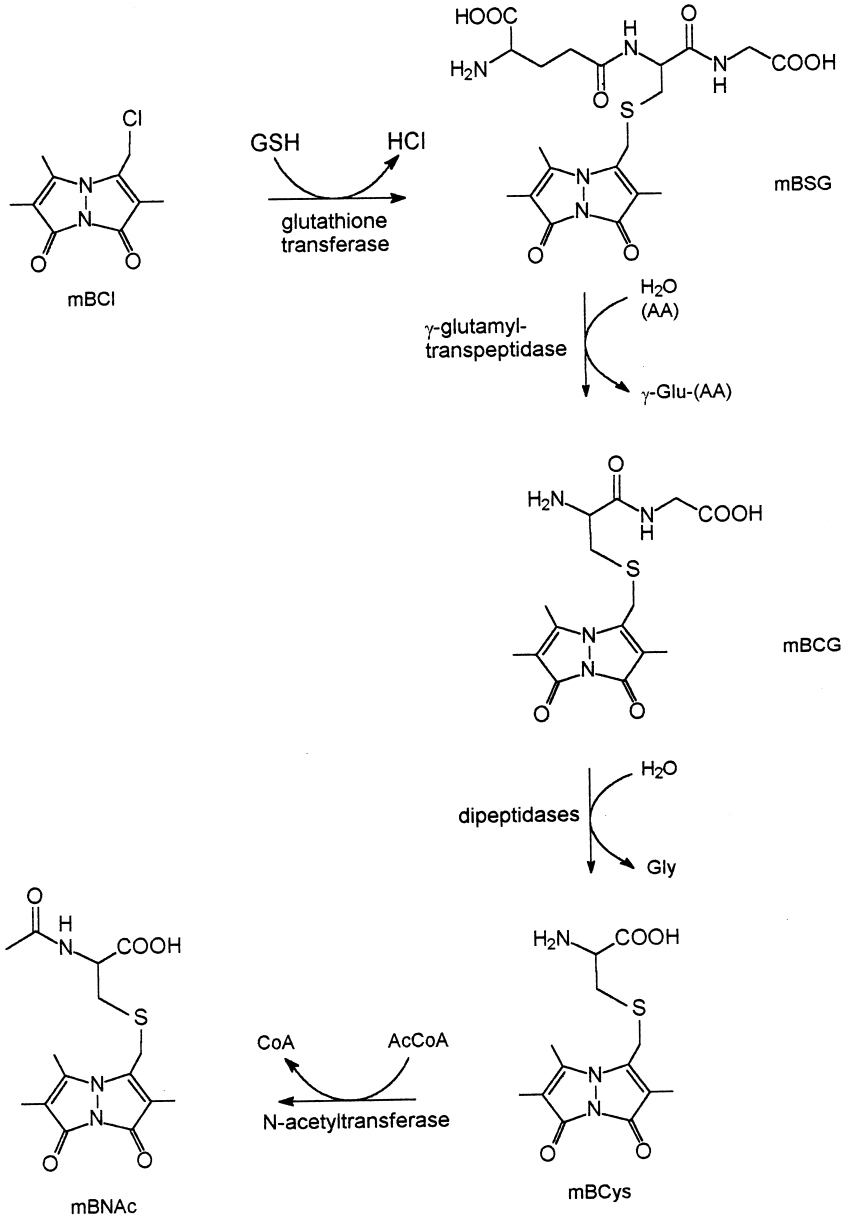

FIG. 1. Mercapturic acid pathway with mBCl as precursor. AA, amino acid as acceptor for the glutamyl moiety.

TABLE 2. Cysteinylglycine S-conjugate dipeptidase activity in different compartments of the rat hepatocyte

	Subcellular Fraction				
				Plasma membranes	
Enzyme	Homogenate	Cytosolic	Microsomal	Sinusoidal	Canalicular
LDH	2740 ± 150	9200 ± 1517 (3.4)	51.0 ± 25.0 (0.02)	—	—
NADPH-cytc reductase	33.0 ± 2.3	ND	194.0 ± 11.0 (5.9)	3.6 ± 0.1 (0.1)	18.0 ± 4.7 (0.5)
Na, K-ATPase	18.0 ± 3.5	1.4 ± 0.7 (0.08)	6.4 ± 3.2 (0.5)	173.0 ± 7.6 (8.8)	ND
APM	14.0 ± 1.5	6.0 ± 0.8 (0.4)	23.0 ± 2.1 (1.7)	18.0 ± 2.2 (1.3)	252 ± 32 (18)
γ-GT	2.5 ± 1.1	ND	2.3 ± 0.2 (0.9)	9.0 ± 3.2 (3.6)	40.0 ± 12.3 (16)
γ-GT (mBSG)	0.73 ± 0.04	ND	0.3 ± 0.1 (0.4)	1.2 ± 0.2 (1.6)	13.0 ± 3.2 (18)
mBCG-dipeptidase	6.1 ± 0.9	21.0 ± 3.7 (3.5)	5.3 ± 1.0 (0.9)	0.69 ± 0.1 (0.1)	11.0 ± 1.7 (1.9)

LDH, lactate dehydrogenase; APM, aminopeptidase M; γ-GT, γ-glutamyltransferase; mBCG-dipeptidase, dipeptidase activity measured with mBCG as substrate. Values are means ± SEM (N = 4–8) and expressed in nmol × min^{-1} × mg of $protein^{-1}$. Enrichment factors in parentheses. ND: not detectable.

with previous studies [31, 32], we observed that the γ-GT activity in the liver of the rat was located mainly on the canalicular domain of the plasma membrane (Table 2). A 20-fold enrichment was measured in the canalicular plasma membrane fraction both with γ-glutamyl-3-carboxy-p-nitroanilide and the bimane glutathione S-conjugate as substrate. No significant enrichment was seen in the sinusoidal and microsomal fractions. This distribution was similar to that of the canalicular marker enzyme APM.

Interestingly, the main activity of the cysteinylglycine S-conjugate dipeptidase was confined to the cytosolic fraction with an enrichment factor of 3.5, equal to that for the cytosolic marker enzyme lactate dehydrogenase (Table 2). Modest enrichment of this enzyme appeared in the canalicular, and none in the sinusoidal and microsomal fractions. Thus, although some dipeptidase activity was detectable as being membrane-bound, its significance for the hydrolysis of the bimane S-conjugate and possibly others is jeopardized in view of the preponderant activity in the soluble fraction of the cell.

Subcellular Distribution of γ-GT and Cysteinylglycine S-Conjugate Dipeptidase Activities in Guinea Pig Liver

Results obtained on cysteinylglycine S-conjugate dipeptidase with guinea pig liver were similar to those described above for the rat (Table 3). Highest specific activity was found in the cytosolic fraction. No clear enrichment of the dipeptidase as compared to the marker enzymes was seen in

TABLE 3. Cysteinylglycine S-conjugate dipeptidase activity in different compartments of the guinea-pig hepatocyte

	Subcellular Fraction				
				Plasma membranes	
Enzyme	Homogenate	Cytosolic	Microsomal	Sinusoidal	Canalicular
LDH	600.0 ± 120	980.0 ± 160 (1.60)	33.0 ± 7.7 (0.07)	—	—
NADPH-cytc reductase	195.0 ± 45.0	4.5 ± 2.3 (0.02)	858.0 ± 266 (4.4)	12.0 ± 2.9 (0.06)	162.0 ± 60.0 (0.8)
Na, K-ATPase	14.0 ± 2.7	ND	4.9 ± 2.4 (0.3)	64.0 ± 13.0 (4.5)	ND
APM	17.0 ± 0.8	13.0 ± 0.8 (0.8)	23.0 ± 1.7 (1.3)	48.0 ± 9.1 (2.8)	125.0 ± 14.0 (7.4)
γ-GT	17.0 ± 2.5	0.5 ± 0.1 (0.03)	37.0 ± 5.3 (2.2)	47.0 ± 9.4 (2.8)	347.0 ± 105.0 (21)
γ-GT (mBSG)	3.5 ± 0.2	ND	3.3 ± 0.2 (0.9)	7.0 ± 1.8 (2.0)	69.0 ± 11.0 (20)
mBCG-dipeptidase	2.7 ± 0.8	5.2 ± 1.4 (1.9)	0.54 ± 0.2 (0.2)	1.1 ± 0.3 (0.4)	3.5 ± 0.5 (1.3)

LDH, lactate dehydrogenase; APM, aminopeptidase M; γ-GT, γ-glutamyltransferase; mBCG-dipeptidase, dipeptidase activity measured with mBCG as substrate. Values are means ± SEM (N = 4–8) and expressed in nmol × min^{-1} × mg of $protein^{-1}$. Enrichment factors in parentheses. ND: not detectable.

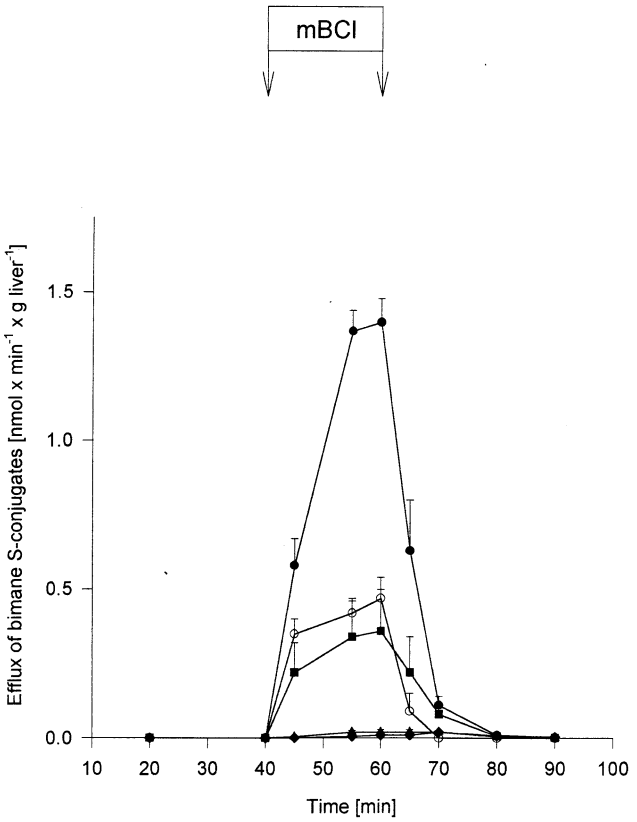

FIG. 2. Appearance of the bimane S-conjugates in bile (closed symbols) and in perfusate (open symbols) formed during perfusion with mBCl. mBCl (0.7 μM; 2.2 nmol × min^{-1} × g of $liver^{-1}$) was infused between 40 and 60 min. The S-conjugates which appear are mBSG (circles), mBCG (squares), mBCys (triangles), and mBNAc (rhombs). Values are means ± SEM (N = 3).

sinusoidal and canalicular plasma membranes. Na,K-ATPase and aminopeptidase M activity measurements indicate that a separation of these membrane fractions was achieved, and that the isolation procedure described for the rat [21] was applicable to guinea pig as well.

Hepatic γ-GT activity is known to be much higher in guinea pig than in rat. This study indicates that the higher level pertained to both the basolateral and the canalicular part of the plasma membrane (Table 3). The canalicular activity exceeded the cysteinylglycine S-conjugate dipeptidase activity by *ca.* 20-fold in this compartment.

In Situ *Rat Liver Perfusions*

Monochlorobimane, infused at 0.7 μM concentration, was almost quantitatively S-conjugated, reaching a steady state after a few minutes (Fig. 2). Eighty percent of the reaction products was recovered from the bile, and 20% from the effluent perfusate (Table 4). mBSG and mBCG were the major products excreted in bile (1.39 and 0.33 nmol × min^{-1} × g of $liver^{-1}$, respectively), in which only minor

TABLE 4. Appearances of bimane S-conjugates during *in situ* perfusions of rat livers with monochlorobimane (mBCl), bimane S-cysteinylglycine (mBCG), and bimane S-cysteine (mBCys)

Influent	Uptake/ percent	mBSG		mBCG		mBCys		mBNAc		Σ
		Bile	Perfusate	Bile	Perfusate	Bile	Perfusate	Bile	Perfusate	
mBCl (2.23)	100	1.39 ± 0.08	0.45 ± 0.05	0.33 ± 0.12	<0.001	0.02 ± 0.008	<0.001	0.01 ± 0.004	<0.001	2.20 ± 0.26
mBCG (2.33)	23–30	0.002 ± 0.0004	<0.001	0.007 ± 0.001	1.63 ± 0.18	0.006 ± 0.001	0.16 ± 0.03	0.22 ± 0.01	0.25 ± 0.04	2.28 ± 0.25
mBCys (3.60)	35	<0.001	<0.001	<0.001	<0.001	0.04 ± 0.03	2.21 ± 0.34	0.52 ± 0.12	0.48 ± 0.05	3.25 ± 0.34

Values are means ± SEM (N = 3–5) and are expressed in nmol × min^{-1} × g of $liver^{-1}$. mBCl, monochlorobimane; mBSG, bimane S-glutathione; mBCG, bimane S-cysteinylglycine; mBCys, bimane S-cysteine; mBNAc, bimane S-(*N*-acetyl-)cysteine. Recoveries: 92–97%.

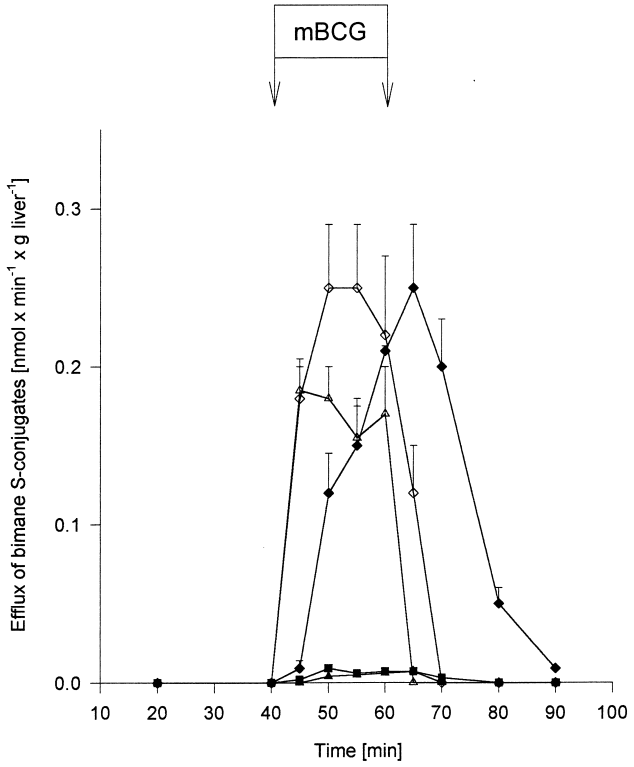

FIG. 3. Appearance of the bimane S-conjugates in bile (closed symbols) and in perfusate (open symbols) formed during perfusion with mBCG. mBCG (0.73 μM; 2.3 nmol × min^{-1} × g of $liver^{-1}$) was infused between 40 and 60 min. The S-conjugates which appear are mBCG (squares), mBCys (triangles), and mBNAc (rhombs). Values are ± SEM (N = 3).

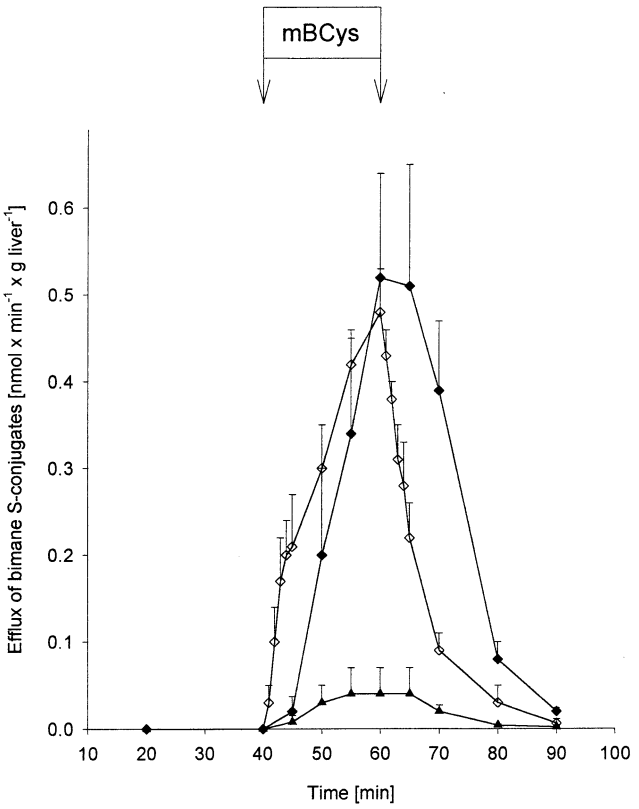

FIG. 4. Appearance of the bimane S-conjugates in bile (closed symbols) and in perfusate (open symbols) formed during perfusion with mBCys. mBCys (1.1 μM; 3.6 nmol × min^{-1} × g of $liver^{-1}$) was infused between 40 and 60 min. The S-conjugates which appear are mBCys (triangles) and mBNAc (rhombs). Values are ± SEM (N = 5).

amounts of mBCys and mBNAc appeared (0.02 and 0.01 nmol × min^{-1} × g of $liver^{-1}$, respectively). In the effluent perfusate, the glutathione S-conjugate was the only detectable product (0.45 nmol × min^{-1} × g of $liver^{-1}$). These data indicate that further degradation of sinusoidal mBSG and biliary mBCG was not efficient in the rat, resulting in a lack of mBNAc formation from mBCl.

To investigate this further, perfusions with different bimane S-conjugates were performed. In line with the limitation of sinusoidal mBSG degradation mentioned above, the glutathione conjugate passed the liver almost unmetabolized upon infusion (data not shown). However, mBCG, infused at 0.7 μM of concentration, was subject to extensive metabolism (Fig. 3, Table 4). Thirty percent of the dipeptide S-conjugate was converted by the liver to form mBNAc as the main product (0.47 nmol × min^{-1} × g of $liver^{-1}$). Lower amounts of the cysteine S-conjugate were released into the effluent perfusate (0.16 nmol × min^{-1} × g of $liver^{-1}$). mBNAc appeared in bile and perfusate in almost equal amounts. Both mBCG and mBCys were hardly detectable in bile (0.007 and 0.006 nmol × min^{-1} × g of $liver^{-1}$, respectively).

Perfusions with mBCys (1.1 μM) showed that this conjugate was taken up and metabolized to approximately the same extent as the dipeptide S-conjugate (Table 4). The mercapturate was rapidly formed in line with results by Inoue *et al.* [33] obtained with S-benzylcysteine. mBNAc was excreted into bile and into the effluent perfusate in almost equal amounts (0.52 and 0.48 nmol × min^{-1} × g of $liver^{-1}$, respectively). Only small amounts of mBCys were found in bile (Table 4 and Fig. 4).

DISCUSSION

Compartmentation of Cysteinylglycine S-Conjugate Dipeptidase

Early studies on the subcellular distribution of dipeptide S-conjugate hydrolyzing activity performed on rat kidney demonstrated the existence of two different brush-border-membrane enzymes with activity toward cysteinylglycine S-conjugates, identified as aminopeptidase M and dehydropeptidase I [14, 15, 27]. While the former was shown to be highly active in kidney, brain and small intestine, the latter showed high activity in lung and kidney [16]. In liver, both enzyme activities were only moderate [16]. Immunohistochemical studies on the dehydropeptidase I indicated both brush border and basolateral localization in kidney [15]. Northern blot analysis revealed the expression of dehydropeptidase I in lung, kidney and small intestine, but not in liver [34, 35]. In our study on rat and guinea pig liver, we found significant cysteinylglycine S-conjugate dipepti-

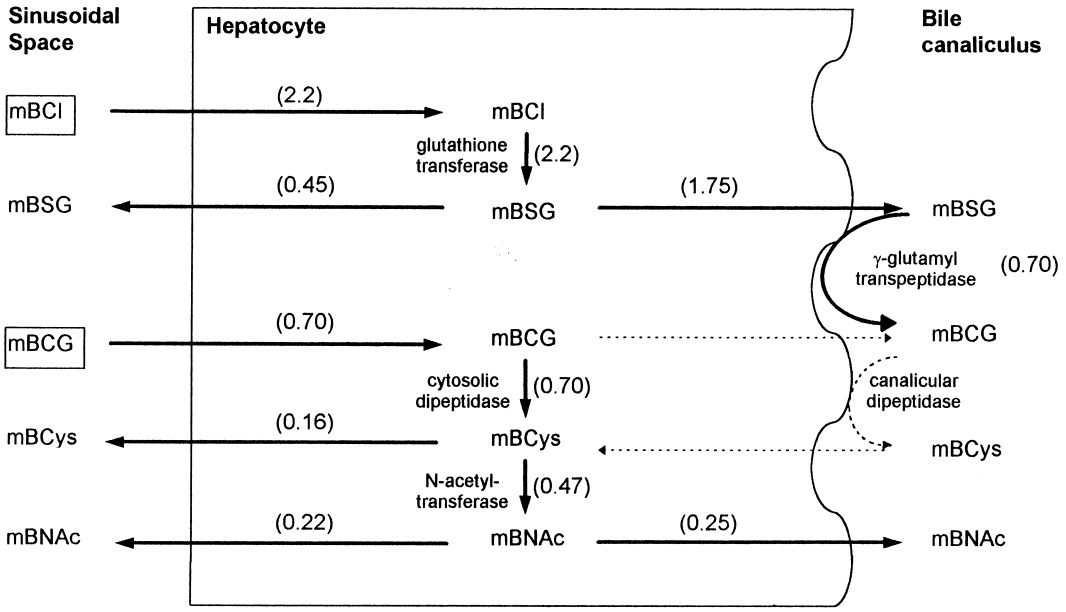

FIG. 5. Major pathways of metabolism of mBCl and mBCG infused in perfused rat liver. Flow rates calculated from Table 4 in parentheses. Dotted lines show the alternative route including canalicular formation and reuptake of the cysteinyl S-conjugate Ref. 3), thought to be of minor importance here.

dase activity in isolated canalicular but not in sinusoidal plasma membranes (Table 2). Unexpectedly, highest specific activity was seen in the cytosolic fraction. As this fraction comprised *ca.* 28% of cellular protein (not shown), the cytosolic activity was calculated to be over 95% of total. Thus, in contrast to kidney [13], the major part of S-conjugate dipeptidase activity in liver was not membrane-bound. This was also observed in guinea pig liver (Table 3).

Perfusion Studies

We further investigated intrahepatic mercapturic acid formation in *in situ* rat liver perfusions (single pass) using monochlorobimane and the S-conjugates derived therefrom. The bimane compounds are highly fluorescent, which allows sensitive detection both in bile and in the effluent perfusate. Monochlorobimane is rapidly and quantitatively S-conjugated within the liver due to the presence of high amounts of GST (Table 1). In accordance with the known preferential excretion of glutathione S-conjugates into bile, approximately 20% of the bimane S-conjugates appeared in the effluent perfusate, almost exclusively as the glutathione S-conjugate, and 80% in bile, in which the glutathione and the cysteinylglycine S-conjugates were the major products (Fig. 2). In spite of the relatively high dipeptidase activity present in the liver (Table 1), there was no significant cysteine S-conjugate and mBNAc formation from monochlorobimane. Similar results were reported previously with 1-chloro-2,4-dinitrobenzene [9]. The lack of further metabolism of cysteinylglycine S-conjugate in bile may be related to the cellular compartmentation of the dipeptidases. Apparently, the intracellular dipeptidase is not accessible to the biliary dipeptide S-conjugate, and the dipeptidase activity in the canalicular compartment is not sufficient to allow for significant hydrolysis.

The hepatic capacity for mercapturic acid formation is evident from perfusions with the cysteinylglycine and cysteine S-conjugates (Figs. 3 and 4). It is noteworthy that during the metabolism of sinusoidal cysteinylglycine S-conjugate, showing substantial mBNAc formation, relatively low amounts of the dipeptide conjugate were detected in bile (Table 4). The presence of a more than 10-fold higher concentration during infusion with mBCl did not lead to significant production of either the cysteine S-conjugate or mBNAc. Thus, when the cysteinylglycine S-conjugate was generated in the canalicular compartment, no significant mBNAc formation was observed. However, when the cysteinylglycine S-conjugate was taken up from the sinusoidal space at the same rate, there was almost complete conversion of the dipeptide, mainly to mBNAc. This shows that the metabolic fate of the intracellular cysteinylglycine S-conjugate is different from the canalicular, and strongly suggests that the metabolism of the sinusoidal cysteinylglycine S-conjugate does not involve canalicular hydrolysis. For this purpose, a function of the cytosolic enzyme is postulated (Fig. 5).

Dipeptidase activity in the sinusoidal plasma membrane (Table 2) is too low to account for significant extracellular degradation in our perfusion studies. Assuming that plasma membrane protein amounts to *ca.* 5% of total cellular protein [36] we calculated sinusoidal hydrolysis of the cysteinylglycine S-conjugate at 1 μM to be less than 1% of the observed hepatic dipeptide metabolism. This means that cellular uptake of the intact dipeptide occurs rather than sinusoidal degradation followed by uptake of the cysteine S-conjugate.

Intra- and Interorgan Mercapturic Acid Formation

It has been suggested that γ-GT activity is limiting for intrahepatic mercapturic acid formation in rat liver and that the more efficient formation in the guinea pig is due to the much higher activity of the enzyme in this species. Sinusoidal activity may play a key role in this respect [3, 37]. Our data support this contention, as both in guinea pig and in rat canalicular transpeptidase is not, or only partly, a limiting step (Tables 2 and 3). An intrahepatic mercapturic acid pathway could also be of great importance for humans, who possess high γ-GT on the sinusoidal side of the hepatocyte [38].

An additional important factor would be the partitioning of glutathione S-conjugate excretion. In the rat, the major part is transported into bile, and only low amounts into the sinusoidal space [5]. In other species this is less clear. Two different glutathione S-conjugate transporters have been identified, designated MRP1 and MRP2 and belonging to the ATP-binding cassette family of transporters (39–41; see 42 for recent review). While MRP2 is highly expressed in liver and localized exclusively in the canalicular domain of the plasma membrane [39], MRP1 is thought to possess a more basolateral localization [40, 42], and is expressed in several other tissues [41], functioning as a source of systemic glutathione S-conjugates. Furthermore, the impact of organic anion transport system(s) present in the hepatic plasma membrane remains to be elucidated in different species. Some glutathione S-conjugates, such as the thioether of sulfobromophthalein, are effectively taken up by the liver and possibly influence sinusoidal cysteinylglycine S-conjugate formation and further metabolism to the mercapturic acid.

Inoue et al. [7] pointed out the importance of a hepatorenal interplay for the metabolism of systemic glutathione S-conjugates. I.v. injection of S-carbamide methyl glutathione led to an immediate accumulation in the kidney followed by the appearance of the cysteine and *N*-acetylcysteine S-conjugates in the liver and excretion of mercapturic acid in urine. It was suggested that the kidney is a major site of transpeptidation and dipeptide hydrolysis, while the liver is the major site of cysteine S-conjugate clearance and *N*-acetylation, in spite of the higher specific activity of *N*-acetyltransferase in kidney [7]. Our results show that not only cysteine S-conjugates, but also cysteinylglycine S-conjugates, which may escape renal dipeptidases [43], may be effectively taken up and metabolized by the liver (Fig. 5).

In conclusion, the work presented demonstrates the existence of substantial cysteinylglycine S-conjugate dipeptidase activity in the cytosol of the liver, as observed in rat and guinea pig. The enzyme may have a central function in intrahepatic and interorgan mercapturic acid formation from glutathione S-conjugates, in that it participates in the metabolism of systemic cysteinylglycine S-conjugates. These are produced by hepatic sinusoidal γ-GT, low in the rat but high in guinea pig and human, or may reach the liver from other tissues high in γ-GT, such as kidney or intestine. Whether the cytosolic cysteinylglycine S-conjugate dipeptidase is a novel enzyme or one of the known cellular peptidases with broad substrate selectivity is under current investigation.

The skillful technical assistance of Gisela Michael is gratefully acknowledged. The work was supported by a grant from the Deutsche Forschungsgemeinschaft (Grant Ak 8/2–1).

References

1. Stevens JL and Jones DP, The mercapturic acid pathway: biosynthesis, intermediary metabolism and physiological disposition. In: *Coenzymes and Cofactors* (Eds. Dolphin D, Poulson R and Avramovic O), pp. 45–84. John Wiley & Sons, New York, 1989.
2. Dekant W, Lash LH and Anders MW, Formation and fate of nephrotoxic and cytotoxic glutathione S-conjugates: Cysteine conjugate β-lyase pathway. *Adv Pharmacol* **27:** 114–162, 1994.
3. Hinchman CA and Ballatori N, Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. *J Toxicol Environ Health* **41:** 387–409, 1994.
4. Commandeur JNM, Stijntjes GJ and Vermeulen NPE, Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. *Pharmacol Rev* **47:** 271–330, 1995.
5. Wahlländer A and Sies H, Glutathione S-conjugate formation from 1-chloro-2,4-dinitrobenzene and biliary excretion in the perfused rat liver. *Eur J Biochem* **96:** 441–446, 1979.
6. Akerboom TPM, Narayanaswami V, Kunst M and Sies H, ATP-dependent S-(2,4-dinitrophenyl)glutathione transport in canalicular plasma membrane vesicles from rat liver. *J Biol Chem* **266:** 13147–13152, 1991.
7. Inoue M, Okajima K and Morino Y, Metabolic coordination of liver and kidney in mercapturic acid biosynthesis *in vivo*. *Hepatol* **2:** 311–316, 1982.
8. Gietl Y, Vamvakas S and Anders MW, Intestinal absorption of S-(pentachlorobutadienyl)glutathione and S-(pentachlorobutadienyl)-L-cysteine, the glutathione and cysteine S-conjugates of hexachlorobuta-1,3-diene. *Drug Met Disp* **19:** 703–707, 1991.
9. Hinchman CA, Matsumoto H, Simmons TW and Ballatori N, Intrahepatic conversion of a glutathione conjugate to its mercapturic acid: Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J Biol Chem* **266:** 22179–22185, 1991.
10. Hinchman CA, Truong AT and Ballatori N, Hepatic uptake of intact glutathione S-conjugate, inhibition by organic anions and sinusoidal catabolism. *Am J Physiol* **265:** G547–G554, 1993.
11. Hinchman CA and Ballatori N, Glutathione-degrading capacities of liver and kidney in different species. *Biochem Pharmacol* **40:** 1131–1135, 1990.
12. Hughey RP, Rankin BB, Elce JS and Curthoys NP, Specificity of a particulate rat renal peptidase and its localization along with other enzymes of mercapturic acid synthesis. *Arch Biochem Biophys* **186:** 211–217, 1978.
13. McIntire T and Curthoys NP, Renal catabolism of glutathione. *J Biol Chem* **257:** 11915–11921, 1982.
14. Kozak EM and Tate SS, Glutathione-degrading enzymes of microvillus membranes. *J Biol Chem* **257:** 6322–6327, 1982.
15. Hirota T, Nishikawa Y, Tanaka M, Fukuda K, Igarashi T and Kitagawa H, Localization of dehydropeptidase I, an enzyme

processing glutathione, in the rat kidney. *J Biochem* **102:** 547–550, 1987.
16. Hirota T, Nishikawa Y, Tanaka M, Igarashi T and Kitagawa H, Characterization of dehydropeptidase I in the rat lung. *Eur J Biochem* **160:** 521–525, 1986.
17. Briviba K, Fraser G, Sies H and Ketterer B, Distribution of the monochlorobimane-glutathione conjugate between nucleus and cytosol in isolated hepatocytes. *Biochem J* **294:** 631–633, 1993.
18. Ellman GL, Tissue sulfhydryl groups. *Arch Biochem Biophys* **82:** 70–77, 1959.
19. Kosower NS and Kosower EM, Thiol labeling with bromobimanes. *Methods Enzymol* **143:** 77–84, 1987.
20. Cadenas E and Sies H, Low level chemiluminescence of liver microsomal fractions initiated by tert-butylhydroperoxide. *Eur J Biochem* **124:** 349–356, 1982.
21. Akerboom TPM and Sies H, Transport of glutathione disulfide and glutathione S-conjugates in hepatocyte plasma membrane vesicles. *Meth Enzymol* **233:** 416–425, 1994.
22. Akerboom TPM, Bilzer M and Sies H, The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J Biol Chem* **257:** 4248–4252, 1982.
23. Akerboom TPM, Schneider I, vom Dahl S and Sies H, Cholestasis and changes of portal pressure caused by chlorpromazine in the perfused rat liver. *Hepatol* **13:** 216–221, 1991.
24. Bergmeyer HU, Bergmeyer J and Graßl M, In: *Methods of Enzymatic Analysis*, *Meth Enz Anal* **3** (3rd edition), pp. 118–126. Verlag Chemie, Weinheim, 1983.
25. Sottocasa GL, Kuylenstierna B, Ernster L and Bergstrand A, An electron-transport system associated with the outer membrane of liver mitochondria. *J Cell Biol* **32:** 415–438, 1967.
26. Scharschmidt BF, Keefe EB, Blankenship NM and Ockner RK, Validation of a recording spectrophotometric method for measurement of membrane-associated Mg- and Na,K-ATPase activity. *J Lab Clin Med* **93:** 790–799, 1979.
27. Rankin BB, McIntire TM and Curthoys NP, Brush border membrane hydrolysis of S-benzylcysteine-*p*-nitroanilide, an activity of aminopeptidase M. *Biochem Biophys Res Com* **96:** 991–996, 1980.
28. Orlowski M and Meister A, γ-Glutamyl-*p*-nitroanilide: a new convenient substrate for determination and study of L- and D-γ-glutamyltranspeptidase activities. *Biochim Biophys Acta* **73:** 679–681, 1963.
29. Habig WH, Pabst MJ and Jakoby WB, Glutathione S-transferases. *J Biol Chem* **249:** 7130–7139, 1974.
30. Bensadoun A and Weinstein D, Assay of proteins in the presence of interfering materials. *Anal Biochem* **70:** 241–250, 1976.
31. Meier PJ, Sztul ES, Reuben A and Boyer J, Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. *J Cell Biol* **98:** 991–1000, 1984.
32. Horbach ME, Sies H and Akerboom TPM, Identification of a hepatic plasma membrane glutathione S-transferase activated by *N*-ethylmaleimide. *Biochim Biophys Acta* **1148:** 61–66, 1993.
33. Inoue M, Okajima K and Morino Y, Hepato-renal cooperation in biotransformation, membrane transport and elimination of cysteine S-conjugates of xenobiotics. *J Biochem* **95:** 247–254, 1984.
34. Adachi H, Ishida N and Tsujimoto M, Primary structure of rat renal dipeptidase and expression of its mRNA in rat tissues and COS-1 cells. *Biochim Biophys Acta* **1132:** 311–314, 1992.
35. Habib GM, Barrios R, Shi ZZ and Liebermann MW, Four distinct membrane-bound dipeptidase RNAs are differentially expressed and show discordant regulation with γ-glutamyltranspeptidase. *J Biol Chem* **271:** 16273–16280, 1996.
36. Touster O, Aronson Jr NN, Dulaney JT and Henrickson H, Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J Cell Biol* **47:** 604–618, 1970.
37. Lanca AJ and Israel Y, Histochemical demonstration of sinusoidal γ-glutamyltransferase activity by substrate protection fixation: Comparative studies in rat and guinea pig liver. *Hepatol* **14:** 857–863, 1991.
38. Purucker E and Wernze H, Hepatic efflux and renal extraction of plasma glutathione: Marked differences between healthy subjects and the rat. *Klin Wochenschr* **68:** 1008–1012, 1990.
39. Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T and Keppler D, cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J Biol Chem* **271:** 15091–15098, 1996.
40. Paulusma CC, Bosma PJ, Zaman GJR, Bakker CTM, Otter M, Scheffer GL, Scheper RJ, Borst P and Oude Elferink RPJ, Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271:** 1126–1128, 1996.
41. Flens MJ, Zaman GJR, van der Valk P, Izquierdo MA, Schoeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJLM and Scheper RJ, Tissue distribution of the multidrug resistance protein. *Am J Pathol* **148:** 1237–1247, 1996.
42. Keppler D, Leier I and Jedlitschky G, Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biol Chem* **378:** 787–791, 1997.
43. Snel CAW, Moons MM, Russel FGM and Mulder GJ, Disposition of the bromosulfophthalein-glutathione conjugate in the isolated perfused rat kidney. *J Pharmacol Exp Ther* **273:** 1300–1306, 1995.