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STEREOSELECTIVE CONJUGATION OF 2-BROMOCARBOXYLIC ACIDS AND THEIR UREA DERIVATIVES BY RAT LIVER GLUTATHIONE TRANSFERASE 12-12 AND SOME OTHER ISOFORMS

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Abstract—Glutathione (GSH) conjugation of the separate enantiomers of five 2-bromocarboxylic acids and some of their urea derivatives by rat liver GSH transferases (GSTs) was studied. The liver cytosolic fraction conjugated all compounds, except for (*R*)-2-bromoisovaleric acid, with a variable degree of stereoselectivity. A GST pool, prepared by *S*-hexyl-GSH affinity chromatography, conjugated the urea derivatives at a somewhat higher rate but had very little activity towards the carboxylic acids, indicating that much activity towards the latter substrates was due to transferases not bound by the affinity column. Therefore, the activity was studied of some pure GSTs that are bound only slightly by the affinity column towards the separate enantiomers of 2-bromovaleric acid (BV), its urea derivative and 2-bromo-3-phenylpropionic acid (BPP). No activity was detected with transferases 5-5 and 8-8. Transferase 1-1 was active towards all compounds with high activity towards the urea derivatives. Transferase 12-12 showed high, stereospecific activity towards the *R* enantiomers of BV, its urea derivative and BPP.

Glutathione (GSH‡) conjugation, catalysed by the glutathione transferases (GSTs), is an important pathway in the detoxication of many xenobiotics [1]. The cytosolic transferases can be divided into four classes: α, μ, π and θ [2–4] that catalyse the reaction of different substrates with GSH, although there is some overlapping. In addition, there is a microsomal GST [5].

Stereoselectivity of GSTs towards chiral substrates has been studied in our group with the chiral substrate, 2-bromoisovalerylurea (BIU), following the finding in the rat *in vivo* that the conjugation of (*R*)-BIU was more rapid than that of (*S*)-BIU [6]. The GSH conjugation of (*R*)-BIU was mainly catalysed by rat liver μ-isoenzymes and that of (*S*)-BIU by α-isoenzymes, but some overlap was found [7]. *In vivo*, BIU can be hydrolysed to chiral 2-bromoisovaleric acid (BI) by amidase activity. Both in the rat *in vivo* and in *in vitro* preparations, only (*S*)-BI was conjugated with GSH. *In vitro* experiments with purified isoenzymes showed that this conjugation was catalysed by the α-isoenzyme 2-2 [7]. Furthermore, the GST isoenzymes were more active towards BIU than BI.

Because we are studying structure–activity relationships of GSH conjugation towards 2-bromocarboxylic acids, we wanted to find out whether this stereoselectivity in GSH conjugation towards BIU and BI applies to other 2-bromocarboxylic acids as well. The compounds used in this study are the separate enantiomers of 2-bromovaleric acid (BV), 2-bromo-3-methylvaleric acid (BMV) and 2-bromo-3-phenylproprionic acid (BPP) and the urea derivative 2-bromovalerylurea (BVU) (Fig. 1). Both BMV and BMVU, have *two* chiral centers, so that the effect of introduction of an additional chiral center can be studied. We were particularly interested in the GST activities that were not retained by the S-hexyl-GSH affinity column.

MATERIALS AND METHODS

Materials. The synthesis of the separate enantiomers of BMV and BPP has been described elsewhere [8, 9]. Briefly, for the preparation of the separate enantiomers of the 2-bromocarboxylic acids from the corresponding 2-amino acids, the enantioselective method of Briggs and Morley [10] was used with minor modifications. The 2-bromocarboxylic acids were subsequently converted to their ureides as described by te Koppele *et al.* [6]. BV and its urea derivative (BVU) were prepared likewise. Chemical purities, based on the area under the curve of the HPLC chromatogram with UV detection at 210 nm, were: (*R*)-BV, 97%; (*S*)-BV, 76%; (*R*)-BVU and (*S*)-BVU, 98%; (2*R*, 3*R*)- and (2*R*, 3*S*)-BMV, 87%; (2*S*, 3*S*)-BMV, 86%; (2*S*, 3*R*)-BMV, 88%; (*R*)-BPP and (*R*)-BPPU, 92%; (*S*)-BPP and (*S*)-BPPU, 89%; and those of the BI and BIU enantiomers were as reported previously [6]. Enantiomeric purity was 97–99% for all enantiomers,

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‡ Abbreviations: GSH, glutathione; GST, glutathione transferase; S100, cytosolic fraction; BI, 2-bromoisovaleric acid; BIU, 2-bromoisovalerylurea; BV, 2-bromovaleric acid; BVU, 2-bromovalerylurea; BMV, 2-bromo-3-methylvaleric acid; BMVU, 2-bromo-3-methylvalerylurea; BPP, 2-bromo-3-phenylpropionic acid; BPPU, 2-bromo-3-phenylpropionylurea.

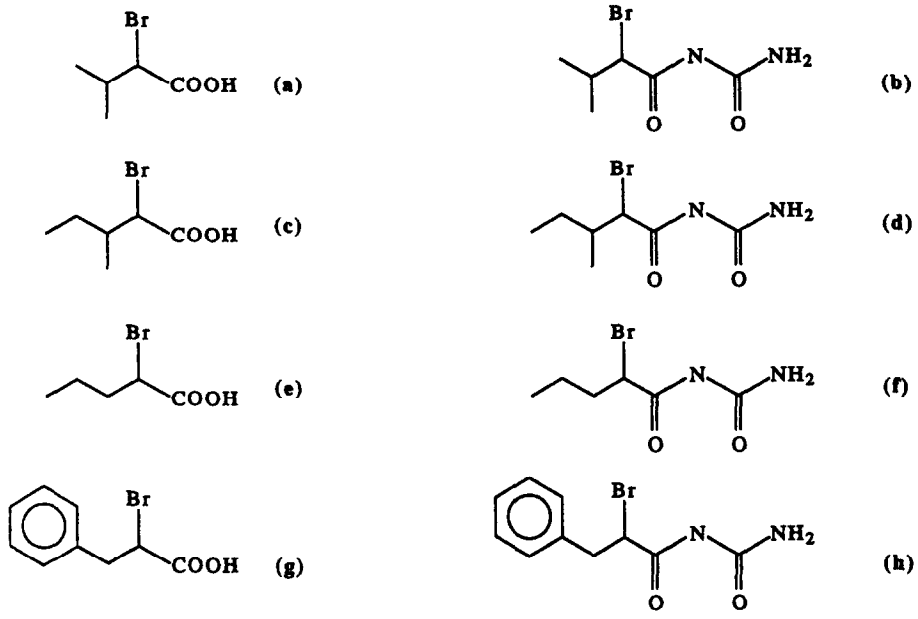

Fig. 1. Structures of: (a) BI; (b) BIU; (c) BMV; (d) BMVU; (e) BV; (f) BVU; (g) BPP; (h) BPPU.

except (*R*)-BPPU (79%) and (*S*)-BPPU (76%). The BPPU enantiomers contained approximately 20% of the other enantiomer which is due to racemization during the urea derivatization.

In order to quantitate the GSH conjugates formed from BV and BVU, these conjugates were chemically synthesized. Equimolar amounts of GSH and (*RS*)-BV or (*RS*)-BVU were dissolved in a mixture of water at pH 10 (adjusted with NaOH):MeOH, 1:1 (v/v); final concentrations of (*RS*)-BV and (*RS*)-BVU were 0.33 and 0.16 M, respectively. For both compounds the mixture was refluxed for 1 hr at 75°, whereafter the methanol was evaporated. The residue was acidified to pH 2 (with HCl) and the GSH conjugates were further purified by preparative column HPLC. Elution started with 0.1% (v/v) trifluoroacetic acid in water. The eluent was changed gradually in 30 min to 30% acetonitrile and 70% 0.1% (v/v) trifluoroacetic acid. Fractions containing the GSH conjugates of (*RS*)-BV or (*RS*)-BVU were collected and lyophilized.

BMV and BMVU contain two chiral carbon atoms: C2 and C3; the structure of a stereoisomer will be given as, for example, (2*R*, 3*S*) which means that the C-2 carbon has the *R* configuration and the C-3 carbon the *S* configuration.

Preparation of rat liver cytosol and a GST pool by S-hexyl-GSH affinity column chromatography. Male Wistar rats from the Sylvius Laboratories, University of Leiden (SPF, 200–250 g body weight) were used. After decapitation, a 33% (w/v) liver homogenate was made in 0.15 M potassium chloride. The cytosolic fraction (S100) was prepared by centrifugation of this homogenate for 20 min at 9000 *g* and subsequent centrifugation of the supernatant for 75 min at 100,000 *g*. The S100 was used to prepare a GST pool by *S*-hexyl-GSH affinity chromatography as described by Guthenberg *et al.* [11]. The enzyme fractions were stored at −80°. The specific activity of the different GST-containing fractions towards 1-chloro-2,4-dinitrobenzene was measured according to the method of Habig *et al.* [12]. The specific activities were 2.1, 0.4 and 20 U/mg protein for a typical cytosolic fraction, flow-through and GST pool, respectively.

Subunit analysis of the GST pool. The GST subunit composition was determined by reversed phase HPLC as described by Ostlund Farrants *et al.* [13], and modified by Meyer *et al.* [14].

Purified GST. Rat GSTs not retained or only partially retained by *S*-hexyl-GSH agarose were purified as follows. GST 1-1 was prepared according to Beale *et al.* [15], GST 8-8 according to Meyer *et al.* [14] and GSTs 5-5 and 12-12 according to Meyer *et al.* [4].

Enzyme assays. The incubations were performed in a 50 mM potassium phosphate buffer, pH 7.4. Final concentrations were 5 mM GSH and 1 mM acceptor substrate. The separate enantiomers were dissolved in dimethyl sulphoxide: the final concentration of dimethyl sulphoxide in the incubation mixture did not exceed 3% (v/v). The protein concentrations were different for the various incubations. For GST 5-5 and 12-12, 3 and 10 μg protein/mL, respectively, were used. For GST 1-1, 8-8 and the GST pool the protein concentration was 0.2; 0.3 and 0.5 mg/mL, respectively, and in the S100 assays it was 6 mg/mL. Total incubation volume was 60 μL. Incubation time was 3 min for the urea derivatives and 5 min for the 2-bromocarboxylic acids. The reaction was stopped by adding an equal volume of ice-cold HPLC buffer, consisting of three parts methanol and two parts of an aqueous solution containing 0.1 M $NaNO_3$, 0.01 M KBr and 0.1 M

citric acid (with or without an ion-pair reagent, as mentioned under analysis of the GSH conjugates). GST 12-12 appeared to be remarkably stable: during 30 min of incubation, no decay in GST activity was observed.

Analysis of the GSH conjugates of BI, BV, BMV, BPP and their urea derivatives. The GSH conjugates in the incubation mixtures were measured by HPLC with an electrochemical detection system as described elsewhere [16], using a Spherisorb ODS II (5 μm particles; 15 × 0.3 cm) column. The GSH conjugates of BI, BV, BMV, BIU, BVU and BMVU were analysed with an eluent consisting of four parts of an aqueous solution containing 0.1 M $NaNO_3$, 0.01 M KBr, 0.01 M citric acid and 0.1 mM sodium decanesulphonate (as ion-pair reagent), and one part MeOH at a flow rate of 0.45 mL/min [8, 17]. (*S*)-IU-G, the glutathione conjugate of (*R*)-BIU, was used as calibration (external) standard [18]. For the quantitation of the GSH conjugates formed from BV and BVU, the same external standard was used. The chemically synthesized GSH conjugates of BV and BVU were used for calibration. The conjugates of BPP and BPPU were analysed with the same eluent but in this case the ion-pair reagent was omitted [9]; the calibration standard used was (*R*)-IU-MA, the mercapturate of (*S*)-BIU [18]. As yet, no quantitative calibration is possible for BPP and BPPU; therefore, only some qualitative preliminary data will be presented.

RESULTS

GSH conjugation by liver cytosol (S100) and the GST pool obtained by S-hexyl-GSH affinity chromatography

When the substrates were incubated with GSH and S100 as a source of GST, almost all substrates were conjugated to at least some extent (Table 1). In general, the rate of conjugation of the 2-bromocarboxylic acids was much lower than that of the corresponding urea derivatives. Preliminary data (not shown) on BMVU and BPPU confirmed this (*R*)-BI was the only compound without detectable conjugation.

When the GST pool purified by affinity chromatography was used, the expected increase in specific activity was found only for the BVU enantiomers. Preliminary data on (2*S*, 3*S*)-BMVU, (2*S*, 3*R*)-BMVU and BPPU showed a similar 2–10-fold increase in activity in the GST pool. However, towards the 2-bromocarboxylic acids most or all of the activity was lost in this GST pool (Table 1). The subunit composition of the GST pool showed that subunit 3 was the major component of the GST pool, representing 45% of the total amount of transferases. The subunits 2 and 4 were present in almost equal amounts: 21% and 26%, respectively. Subunit 1 was a minor component, representing 8%.

The flow-through contained GST activity towards most of the substrates, but, in most cases, the activity was lower than that in the S100 (data not shown).

GSH conjugation by some pure GST enzyme forms

The above experiments suggested that the GSTs responsible for the GSH conjugation of 2-bromocarboxylic acids were largely not retained by the *S*-hexyl-GSH affinity column. Therefore, the GST forms that are known to be little retained by the *S*-hexyl-GSH affinity column were tested for their activity towards the separate enantiomers of BV, BPP and BVU: 1-1, 5-5, 8-8 and 12-12. Only substrates for which the conjugates could be detected at very low enzyme concentrations were studied due to limited availability of θ class GSTs. All substrates were conjugated with 1-1 (Table 2). There was a big difference in the activity of GST 1-1 towards the 2-bromocarboxylic acids on the one hand and their urea derivatives on the other: the rate towards the

Table 1. Glutathione conjugation rate of 2-bromocarboxylic acids and their urea derivatives by rat liver cytosol and a more concentrated GST pool

Compound	S100	GST pool
CDNB	2100	20,000
(*R*)-BV	1.6	0.1
(*S*)-BV	0.9	0.1
(*R*)-BVU	21	165
(*S*)-BVU	12	63
(*R*)-BPP	2.2	0.2
(*S*)-BPP	4.3	0.2
(*R*)-BI	ND	ND
(*S*)-BI	2.1	ND
(*R*)-BIU	28	21
(*S*)-BIU	5.4	7
(2*R*, 3*R*)-BMV	0.2	ND
(2*S*, 3*S*)-BMV	1.1	ND
(2*R*, 3*S*)-BMV	0.1	ND
(2*R*, 3*R*)-BMV	0.6	ND

Rates are given as nmol per min per mg protein. Substrate concentrations were 1 mM for the acceptor substrates and 5 mM for GSH. Incubation times were 3 and 5 min for the 2-bromocarboxylic acids and their urea derivatives, respectively.

ND, not detectable (<0.01–0.04 nmol/min/mg protein).

CDNB, 1-chloro-2,4-dinitrobenzene.

Table 2. GSH conjugation rate of substrates by some purified GST isoenzymes

	GST isoenzyme	
Compound	1-1	12-12
(*R*)-BV	1.1	500
(*S*)-BV	2.3	ND*
(*R*)-BVU	232	1300
(*S*)-BVU	82	ND†
(*R*)-BPP	2.0	36
(*S*)-BPP	1.7	ND*

Rates are given as nmol per min per mg protein. The substrate concentrations were 1 mM for the 2-bromo derivatives and 5 mM for GSH. Incubation times were 3 and 5 min for the 2-bromocarboxylic acids and their urea derivatives, respectively.

Not detectable: * < 5–9 nmol/min/mg protein; † < 60 nmol/min/mg protein.

urea derivatives was at least two orders of magnitude higher than that of the corresponding acids. The same applied to the BP and BPPU enantiomers. Isoenzymes 5-5 and 8-8 did not show any detectable activity towards the substrates used (including BPPU). Detection limits were 0.1–0.3 nmol/min/mg protein for GST 8-8 towards the BV and BPP enantiomers; for GST 5-5 this was 2 nmol/min/mg protein. For the BVU enantiomers, detection limits were higher: 5–9 nmol/min/mg protein for GST 8-8 and 20 nmol/min/mg protein for GST 5-5.

GST 12-12 showed a high degree of stereospecificity: only *R* enantiomers were detectably conjugated. The rates were quite high for this type of substrate. Interestingly, (*R*)-BV was a good substrate; still, (*R*)-BVU showed a 2.5-fold higher rate. In contrast, (*R*)-BPP was conjugated only at a slow rate and (*R*)-BPPU was not conjugated at all by GST 12-12.

DISCUSSION

In the course of investigations into structure–activity relationships of GSH conjugation towards 2-bromocarboxylic acids we studied their conjugation *in vitro* as well as that of some of their urea derivatives. All compounds, except for (*R*)-BI, were conjugated with GSH by the cytosolic fraction of the rat liver. GSH conjugation of the free 2-bromo acids was in most cases stereoselective, but for the BV and BPP enantiomers little stereoselectivity was observed. The cytosol represents a rather complex mixture of many GST forms. The more concentrated GST pool, prepared by affinity chromatography, contains only a part of the transferases present, mainly subunits 2, 3 and 4. All urea derivatives were conjugated by the isoenzymes present in the GST pool, but activity towards the free carboxylic acids (BI, BV, BMV and BPP) was almost completely lost. Apparently, the isoenzymes involved in conversion of the latter do not bind to the *S*-hexyl-GSH affinity column.

Some GSTs have little or no affinity for the *S*-hexyl-GSH column. Transferase 1-1 does not bind completely [19], while GST 8-8 [14, 19, 20], GST 5-5 [21] and GST 12-12 [4, 22] do not bind at all. Therefore, we studied whether these purified isoenzymes might be involved in the GSH conjugation of the free 2-bromocarboxylic acids. No activity was detected with GSTs 5-5 and 8-8, whereas isoenzyme 1-1 was active towards all substrates. GST 12-12 (which appeared to be remarkably stable) was highly stereospecific towards the *R* enantiomers of BV and BVU: the ratios of the specific activities of GST 12-12 toward the *R* enantiomer over that towards the *S* enantiomer were 56 and 21, respectively. This difference is more prounounced than that found for GST 4-4 towards BIU, where the ratio was only 7.5 [7], or towards BPP, where the ratio was 5. Thus, a similar stereospecificity of GST 12-12 (class θ) and the μ class GSTs was observed. Stereospecificity of the μ class towards polycyclic aromatic hydrocarbon epoxides [23, 24], aflatoxin B1-8, 9-oxide [25] and BIV [7] also favours the *R*-configuration. This stereospecificity is in agreement with the similar inhibitor sensitivities of some of these compounds (D. Meyer, unpublished data).

(*R*)-BVU appears to be a very good substrate for GST 12-12. Towards (R)-BPP activity is much lower, but (*R*)-BPPU, its urea derivative, was not conjugated at all by GST 12-12. These results indicate that the presence of a urea moiety in a compound does not necessarily result in a more rapid GSH conjugation by GST 12-12. The activity of GST 12-12 towards (*R*)-BV and (*R*)-BVU was comparable to that found towards other substrates like 1,2-epoxy-3(*p*-nitrophenoxy)propane (0.5 μmol/min/mg protein), menaphthyl sulphate (0.5 μmol/min/mg protein) and cumene hydroperoxide (1.5 μmol/min/mg protein) [4, 22]. Since the conjugation rate of the 2-bromocarboxylic acids and their urea derivatives by the other GSTs is low [1], the activity of GST 12-12 towards the (*R*)-2-bromo-derivatives may be an important contribution to the overall hepatic GSH conjugation.

In conclusion, the GSH conjugation of 2-bromocarboxylic acids is not generally stereospecific. However, catalytic activity of GST 12-12 is virtually stereospecific towards the *R* enantiomers tested.

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