Stereoselectivity of rat liver glutathione transferase isoenzymes for α -bromoisovaleric acid and α -bromoisovalerylurea enantiomers

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The stereoselectivity of purified rat GSH transferases towards α -bromoisovaleric acid (BI) and its amide derivative α -bromoisovalerylurea (BIU) was investigated. GSH transferase 2–2 was the only enzyme to catalyse the conjugation of BI and was selective for the (*S*)-enantiomer. The conjugation of (*R*)- and (*S*)-BIU was catalysed by the isoenzymes 2–2, 3–3 and 4–4. Transferase 1–1 was less active, and no catalytic activity was observed with transferase 7–7. Isoenzymes 1–1 and 2–2 of the Alpha multigene family preferentially catalysed the conjugation of the (*S*)-enantiomer of BIU (and BI), whereas isoenzymes 3–3 and 4–4 of the Mu multigene family preferred (*R*)-BIU. The opposite stereoselectivity of conjugation of BI and BIU previously observed in isolated rat hepatocytes and the summation of activities of enzymes known to be present in hepatocytes on the basis of present data are in accord.

INTRODUCTION

GSH conjugation plays an important role in the detoxification of many xenobiotics to which living organisms are exposed. To evaluate the efficiency of the conjugation process in animals or man, the substrates α -bromoisovaleric acid (BI) and α -bromoisovalerylurea (BIU, Fig. 1) have been investigated as model substrates for GSH conjugation *in vivo* (te Koppele *et al.*, 1986a,b, 1987, 1988).

Since the α -carbon atom of both BI and BIU is chiral, two diastereoisomeric conjugates can be formed by conjugation with GSH. As was shown previously, the GSH conjugation of BIU was completely stereospecific (te Koppele *et al.*, 1988): (*R*)-BIU resulted exclusively in the diastereomeric GSH conjugate IU-S-G/I, and (*S*)-BIU was converted exclusively into the other diastereoisomer, IU-S-G/II. Furthermore, stereoselectivity was observed in the rate of conjugate formation: in isolated rat hepatocytes and in intact rats metabolism of (*R*)-BIU was 2–3-fold more rapid than that of (*S*)-BIU (te Koppele *et al.*, 1987, 1988). Stereoselective GSH conjugation was also observed with BI as acceptor substrate (te Koppele *et al.*, 1988). Stereoselectivity in GSH conjugation of enantiomers has also been observed with other

chiral substrates such as epoxides (Cobb *et al.*, 1983; Boehlert & Armstrong, 1984; Dostal *et al.*, 1986) and aralkyl halides (Mangold & Abdel-Monem, 1980, 1983), although it did not occur for the enantiomers of 2-iodooctane and 2-bromo-octane (Ridgewell & Abdel-Monem, 1987).

Surprisingly, in isolated hepatocytes the stereoselectivity of BI conjugation was opposite to that of BIU: (*R*)-BI was conjugated more slowly than (*S*)-BI whereas (*R*)-BIU [with the same spatial arrangement at the chiral centre as (*R*)-BI] was conjugated faster than (*S*)-BIU (te Koppele *et al.*, 1987, 1988). In intact hepatocytes a mixture of transferase isoenzymes is present, however, and differences in stereoselectivity between the isoenzymes in the GSH conjugation of the BI and BIU enantiomers might be the cause of the observed difference in stereoselectivity. In order to investigate this, the GSH conjugation of BI and BIU enantiomers was studied with the purified homodimeric rat GSH transferase isoenzymes 1–1, 2–2, 3–3, 4–4 and 7–7.

MATERIALS AND METHODS

Chemicals

BI, BIU and their separate enantiomers were obtained as described previously (te Koppele *et al.*, 1988). GSH from Boehringer (Mannheim, Germany) was used. Reference compounds for h.p.l.c. were prepared as described previously (te Koppele *et al.*, 1986a).

GSH transferase isoenzymes

Rat liver GSH transferase isoenzymes 1–1, 2–2, 3–3 and 4–4 were prepared from rat liver soluble supernatant fraction as reported previously (Beale *et al.*, 1983); GSH transferase 7–7 was prepared from rat kidney as described

Fig. 1. Structures of α -bromoisovaleric acid (BI) and α -bromoisovalerylurea (BIU)

Abbreviations used: BI, α -bromoisovaleric acid; BIU, α -bromoisovalerylurea; I-S-G, α -(glutathion-S-yl)isovaleric acid; IU-S-G/I and IU-S-G/II, α -(glutathion-S-yl)isovalerylurea diastereoisomers I and II.

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by Meyer *et al.* (1985). Enzyme preparations were dialysed against 5 mM-sodium phosphate buffer, pH 7.4, before use.

Incubation conditions

Incubations were performed at 37 °C in 50 mM-sodium phosphate buffer, pH 7.4. Incubations contained (final concentrations) 5.0 mM-GSH and 2.0 mM acceptor substrate with a protein concentration between 0.2 and 1.5 mg/ml. After preincubation at 37 °C for 2 min the reaction was started by addition of the acceptor substrate (4 mM-BIU and 10 mM-BI stock solutions in 50 mM-sodium phosphate buffer, pH 7.4). Total incubation volumes were 40 or 80 μ l. After incubation for 2.5 min the reaction was stopped by addition of an equal volume of CSPA solution [an aqueous solution of 0.1 M-citric acid/0.1 M- NaNO_3 /0.01 M-KBr/0.1 mM-sodium 1-decanesulphonate (2 parts) to which methanol (3 parts) was added] and the mixture was placed on ice. Samples were stored at -20 °C until analysis. All incubations were performed in duplicate; enzymic data were corrected for non-enzymic conjugation (generally less than 5% of the totally formed conjugates; see below).

Assays

H.p.l.c. analysis of incubation samples (5 μ l) to quantify the GSH conjugates of BIU and BI (IU-S-G and I-S-G respectively) was performed as described elsewhere (te Koppele *et al.*, 1987, 1988). Samples were chromatographed with an isocratic solvent consisting of 85% (v/v) aqueous buffer, pH 2.5 (0.01 M-citric acid/0.1 M- NaNO_3 /0.01 M-KBr/0.1 mM-sodium 1-decanesulphonate), and 15% (v/v) methanol. Retention times of IU-S-G/I, IU-S-G/II and I-S-G were 23.5, 27.3 and 29.2 min respectively. IU-S-G/I and II were quantified by the use of synthetic reference compounds. H.p.l.c. quantification of I-S-G was based on [glutathionyl- ^3H]-I-S-G of known specific radioactivity. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Data are presented as means $\pm$ S.E.M. Kinetic data were analysed by linear regression of Lineweaver-Burk plots. Catalytic efficiency ($k_{\text{cat.}}/K_m = V_{\text{max.}}/[E] \cdot K_m$) values were obtained by dividing the initial velocity (v) by the product of the substrate concentration $[S]$ and total enzyme concentration $[E]$, which is valid at sufficiently low substrate concentrations (Danielson & Mannervik, 1985; Robertson *et al.*, 1986a). At $[S] = 0.5, 0.625$ and 0.83 mM the ratio $v/[S]$ was determined; $k_{\text{cat.}}/K_m = v/[S] \cdot [E]$ was determined by extrapolation to $[S] = 0$. When K_m and $V_{\text{max.}}$ values could be determined the $k_{\text{cat.}}/K_m$ value was calculated directly.

RESULTS

Validation of enzyme assays

The h.p.l.c. system used was sufficiently sensitive to measure low concentrations of GSH conjugates: approx. 1 μ M in the incubation medium. The sensitivity of the electrochemical detection was limited by the large amount of GSH in the incubation mixture, which resulted in a large peak; the GSH conjugates of BI and BIU emerged with its tail (Fig. 2).

The spontaneous non-enzymic conjugation was low, but its contribution to the total conjugation rate varied

Fig. 2. H.p.l.c. chromatograms of deproteinized incubation samples (2 mM-BIU and 5 mM-GSH)

GSH transferase 2-2 (a) without acceptor substrate; (b) with (R)-BIU; (c) with (S)-BIU; (d) with (S)-BI. (e) Reference compounds IU-S-G/I and II, 12.0 and 10.1 μ M respectively.

with the activity of the isoenzyme. With GSH transferase 1-1 spontaneous conjugation of (R)- and (S)-BIU was 15% and 9% of the total amount of conjugate formed respectively. With the more active enzymes the spontaneous reaction accounted for less than 5%.

With all enzymes and all substrates, product formation was linear for at least 2.5 min. Furthermore, the amount of conjugate formed was linear with protein concentration [tested with GSH transferase 4-4 and (R)-BIU, over the range 0.05–0.7 mg of protein/ml]. Initiation of the enzymic reaction with BI or BIU gave the same result as initiation by GSH.

Specific activities of GSH transferase isoenzymes

The activities of purified rat GSH transferases towards the BIU and BI enantiomers were studied. With BI as acceptor substrate, catalysis of GSH conjugation was found only with transferase 2-2 and was stereoselective: (S)-BI was conjugated with GSH but no conjugation of (R)-BI was observed. The enzymic reaction was slow, and spontaneous conjugation was not detectable.

Table 1. Specific activities of rat GSH transferase isoenzymes towards BIU and BI enantiomers

Specific activities were determined by incubation of 2 mM acceptor substrate and 5 mM-GSH. Results are given as means $\pm$ S.E.M. for four duplicate determinations. Abbreviation: N.D., not detectable (≤ 0.2 – 0.4 nmol/min per mg).

GSH transferase	Specific activity (nmol/min per mg of protein)				(R)/(S) ratio
	BI		BIU		
	(R)	(S)	(R)	(S)	
1-1	N.D.	N.D.	5.4±0.3	16.5±1.0	0.33
2-2	N.D.	0.94±0.09	48.6±1.5	138±14	0.35
3-3	N.D.	N.D.	127±4	55.4±2.4	2.3
4-4	N.D.	N.D.	114±8	15.2±1.1	7.5
7-7	N.D.	N.D.	N.D.	N.D.	—

Fig. 3. Representative examples of Lineweaver-Burk plots of GSH transferases 2-2 and 3-3 with the substrates (R)- and (S)-BIU at 5 mM-GSH

(a) GSH transferase 2-2; (b) GSH transferase 3-3. ○, (R)-BIU; ●, (S)-BIU.

Except for transferase 7-7, all isoenzymes tested were active towards the BIU enantiomers. The lowest enzyme activity was observed for isoenzyme 1-1: the enzymic GSH conjugation of (R)-BIU was only 6 times that of the spontaneous reaction. The activity of transferase 1-1 towards (S)-BIU was somewhat higher (Table 1). GSH transferase 2-2 was much more active; substantial activities were observed for both BIU enantiomers. Similarly to transferase 1-1, the rate of transferase 2-2 with (S)-BIU was 2-3 times that with (R)-BIU, which resembles the stereoselectivity of isoenzyme 2-2 towards BI. Substantial enzymic activities towards BIU were also found with transferases 3-3 and 4-4. Interestingly, the stereoselectivity of GSH conjugation of the 3/4 family (the Mu multigene family) was opposite to that of the 1/2 family (the Alpha multigene family); transferase 3-3 as well as transferase 4-4 conjugated (R)-BIU more efficiently than (S)-BIU, whereas transferases 1-1 and 2-2 conjugated (S)-BIU more efficiently than (R)-BIU. The stereoselectivity of transferase 4-4 was more pronounced than that of transferase 3-3; specific

activities for (R)- and (S)-BIU differed 7.5- and 2.3-fold respectively. Isoenzymes 3-3 and 4-4 had equal activities towards (R)-BIU (Table 1).

Specific activities of a rat liver cytosolic fraction towards (R)- and (S)-BIU were 17.4 and 5.4 nmol/min per mg of cytosolic protein when product formation was measured for 2.5 min at 37 °C (at 2 mM-BIU and 5 mM-GSH). Determination of the GSH transferase subunit composition in this liver cytosolic fraction by the procedure of Ostlund Farrants *et al.* (1987) showed that subunits 1, 2, 3 and 4 represented 3.5, 1.4, 2.8 and 4.3% respectively of the cytosolic protein.

Kinetics of GSH conjugation of BI and BIU

The kinetics of the formation of IU-S-G and I-S-G from BIU and BI enantiomers were studied as a function of BIU and BI concentration. Linear Lineweaver-Burk plots were obtained (Fig. 3; a range of 0.5–4.5 mM acceptor substrate was employed at 5 mM-GSH). Owing to the low activities of some of the isoenzymes their K_m and V_{max} values could not be determined. The highest

Table 2. Enzyme kinetic properties of homodimeric rat GSH transferases with BIU and BI enantiomers

At various initial acceptor substrate concentrations initial rates of GSH conjugate formation were determined. K_m and V_{max} were determined from Lineweaver–Burk plots. Catalytic efficiencies k_{cat}/K_m were determined with the use of V_{max} and K_m or with v and $[S]$ (at low substrate concentrations). Abbreviation: N.D., not determined.

GSH transferase	K_m (mM)		V_{max} (nmol/min per mg of protein)		k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)			
					$V_{max}/[E] \cdot K_m$		$v/[E] \cdot [S]$	
	(R)	(S)	(R)	(S)	(R)	(S)	(R)	(S)
With BIU								
1–1	N.D.	2.0	N.D.	33	N.D.	14	5.3	13
2–2	1.4	0.6	56	119	37	185	36	N.D.
3–3	2.6	3.1	169	56	57	16	58	13
4–4	> 10	> 10	N.D.	N.D.	N.D.	N.D.	24	3
With BI								
2–2		5.0		3.2		0.45		0.50

affinity with BIU was found for GSH transferase 2–2 (Table 2). The K_m values indicate a low binding affinity of the substrates. The affinity of transferase 2–2 for (S)-BIU was higher than that for (R)-BIU. Furthermore, the V_{max} with (S)-BIU was 2-fold higher than that with (R)-BIU (Table 2). Thus the higher catalytic activity of transferase 2–2 towards (S)-BIU as compared with (R)-BIU is due to a higher affinity combined with a higher V_{max} value. The affinity of transferase 1:1 for (S)-BIU also was low: K_m was 2 mM. The amount of conjugate formed from (R)-BIU with transferase 1–1 was too small to enable a determination of K_m and V_{max} .

The affinity of transferase 3–3 for (R)- and (S)-BIU was somewhat less than that of isoenzyme 2–2: K_m values were approx. 3 mM. The V_{max} values were of the same order as for transferase 2–2: 50–100 nmol/min per mg (Table 2). In accordance with the observed specific activities (Table 1) the V_{max} with (R)-BIU was larger than that with the (S)-enantiomer, but the K_m values were similar. Owing to the very low affinity of transferase 4–4 for the BIU enantiomers (K_m higher than 10 mM) and the limited water-solubility of BIU (approx. 7 mM) no reliable K_m and V_{max} values could be determined for isoenzyme 4–4.

Catalytic efficiencies (k_{cat}/K_m) were determined from V_{max} and K_m values, if available. When no K_m and V_{max} values could be determined the catalytic efficiency was calculated as the initial rate (v) divided by the product of the total enzyme concentration $[E]$ and substrate concentration $[S]$. The catalytic efficiencies determined this way agreed well with those determined from K_m and V_{max} values (Table 2). Whereas the (R)-BIU/(S)-BIU ratios of k_{cat}/K_m were 0.4 and 0.2 for transferases 1–1 and 2–2, they were 4 and 8 in case of transferases 3–3 and 4–4. This agrees well with the (R)/(S) ratios of the specific activities (Table 1; 0.33, 0.35, 2.3 and 7.5 respectively).

The low rate of metabolism of (S)-BI with isoenzyme 2–2 was a result of a low affinity and an extremely low capacity (K_m 5 mM; V_{max} 3.2 nmol/min per mg; Table 2).

DISCUSSION

The BI and BIU enantiomers are poor substrates for the GSH transferases: the k_{cat}/K_m values of less than

200 $M^{-1} \cdot s^{-1}$ are extremely low as compared with, e.g., 560 000 and 63 000 for transferase 1–1 with 1-chloro-2,4-dinitrobenzene and ethacrynic acid respectively (Danielson & Mannervik, 1985; Jakoby & Habig, 1980). The K_m values of the GSH transferases towards BIU were high, in particular those of isoenzymes 3–3 and 4–4. This implies that, owing to the limited water-solubility of BIU, most of the specific activities had been determined at substrate concentrations below the K_m . Therefore these specific activities are not a reflection of a maximal enzymic activity but merely a relative value related to substrate concentration.

Previous studies with rat liver cytosolic fractions, isolated rat hepatocytes, the perfused rat liver and the intact rat showed that (R)-BIU was conjugated at a rate 2–3-fold greater than (S)-BIU (te Koppele *et al.*, 1987, 1988). The present results indicate that only GSH transferases 3–3 and 4–4 can be responsible for this stereoselectivity, since the preference of transferases 1–1 and 2–2 for the BIU enantiomers is opposite. With the abundance of rat GSH transferase subunits in the cytosolic fractions and the specific activities towards (R)- and (S)-BIU (Table 1) the rat liver cytosol can be calculated to show a ratio of IU-S-G-/I formed over IU-S-G-/II of approx. 2:1. This agrees reasonably well with the ratio of approx. 3:1 found experimentally.

In the intact rat and in isolated hepatocytes as well as in rat liver subcellular fractions (te Koppele *et al.*, 1986b, 1987, 1988; J. M. te Koppele, M. Polhuijs & G. J. Mulder, unpublished work) an opposite stereoselectivity of BI and BIU was observed. The present data provide an explanation for this at-first-sight surprising finding. Whereas isoenzymes 1–1 and 2–2 of the Alpha multigene family preferentially catalysed the conjugation of the (S) forms of both BI and BIU, isoenzymes 3–3 and 4–4 of the Mu multigene family were stereoselective towards the (R) form. The lack of activity of the isoenzymes with subunits 3 and 4 towards BI and the large contribution of these isoenzymes in the conjugation of BIU in rat hepatocytes or the rat *in vivo* explain the observed opposite stereoselectivity towards BI and BIU in the cytosolic and intact cell systems containing mixtures of the GSH transferase isoenzymes.

The active site of each GSH transferase subunit contains a GSH-binding site (G-site) and a binding

Fig. 4. Schematic representation of possible binding interactions of BIU enantiomers at the H-site of GSH transferase isoenzymes

Hydrophobic and polar binding of BIU at the H-site in relation to the G-site are such that (*S*)-BIU can be conjugated more efficiently than (*R*)-BIU by isoenzymes 1-1 and 2-2 of the Alpha multigene family, whereas preferential GSH conjugation of (*R*)-BIU was observed with isoenzymes 3-3 and 4-4 of the Mu multigene family.

site for the acceptor substrate (H-site) (Danielson & Mannervik, 1985; Mannervik, 1985). However, details of the binding sites are not known.

With respect to its interaction with the H-site, BIU seems to contain three relevant moieties: the polarizable bulky bromine atom, the lipophilic isopropyl group and the polar carbonyl urea (ureide) moiety. Hydrophobic interactions between the isopropyl group and the enzyme may be important in the binding of BIU, similarly to the importance of hydrophobic interactions in the binding of aromatic epoxides (Boehlert & Armstrong, 1984). Furthermore, the ureide moiety most likely forms hydrogen bridges with polar groups at the active site (Mannervik *et al.*, 1978; Carne *et al.*, 1979; Inoue *et al.*, 1981; Schasteen *et al.*, 1983; Awasthi *et al.*, 1987). Assuming such a two-point attachment of BIU in the active site the orientation of the bromine atom is determined by the enantiomeric form of BIU. As depicted in Fig. 4, the configuration of the acceptor substrate in the H-site and its orientation towards the G-site probably are important factors in the selective GSH conjugation of the separate BIU enantiomers. Differences in (polar and/or hydrophobic) binding interactions at the H-site and/or orientation of the H-site towards the G-site may explain the observed opposite stereoselectivity of the Alpha and Mu isoenzymes towards the substrate BIU (Fig. 4). The opposite stereoselectivity in GSH conjugation of aromatic epoxides by cytosolic fractions of spleen and kidney as compared with liver, heart and lung led Dostal *et al.* (1986) to speculate along similar lines.

Most studies concerning stereoselective GSH conjugation were performed with epoxides (Cobb *et al.*, 1983; Boehlert & Armstrong, 1984; Dostal *et al.*, 1986; Robertson *et al.*, 1986a,b; Robertson & Jernström, 1986) mostly with isoenzymes from the 3/4 family, and rarely with halogen-containing substrates. With 1-chloro-1-phenylethane the same stereoselectivity was found with isoenzymes from the 1/2 and the 3/4 family: the ratio (*R*SR)-GSH to (*S*SR)-GSH conjugate was approx. 5:1 with isoenzymes B (1-1 and 1-2), C (3-4) and A (3-3) (Mangold & Abdel-Monem, 1983). More recently, Ridgewell & Abdel-Monem (1987) showed that GSH transferase 1-2 lacked stereoselectivity in the GSH conjugation of 2-bromo-octane or 2-iodo-octane, as did rat liver cytosol. In the present paper a distinct stereoselectivity was observed in the conjugation of BIU with GSH catalysed by GSH transferases 1-1, 2-2, 3-3 and 4-4. Interestingly, the stereoselectivity of transferases 1-1 and 2-2 was opposite to that of transferases 3-3 and 4-4. Although the epoxide substrates are chemically quite different from the halogen-containing substrates, some similarities in the GSH conjugation of BIU and the aromatic epoxides were observed. Both types of substrates required the same spatial arrangement of substituents at the chiral carbon atom for transferases 3-3 and 4-4: GSH conjugation was stereoselective for (*R*)-configured carbon atoms. Furthermore, the stereoselectivity of isoenzyme 4-4 was much more pronounced than that of isoenzyme 3-3 (Boehlert & Armstrong, 1984; Robertson *et al.*, 1986a,b; Robertson & Jernström,

1986). Although the present study reports the first opposite stereoselectivity between different multigene families in the rat (Alpha versus Mu family isoenzymes with a halogenated substrate), opposite stereoselectivities have also been described for human transferases with benzo[a]pyrene 4,5-epoxide (Alpha and Mu family isoenzymes versus Pi family transferases; G. L. Foureman, L. A. Dostal, J. R. Hernandez, J. R. Bend, C. Guthenberg, P. Alin, M. Warholm & B. Mannervik, unpublished work, cited in Mannervik, 1985).

In conclusion, the present results indicate that the apparent stereoselective GSH conjugation of (*R*)-BIU and (*S*)-BI in isolated hepatocytes can be explained by the finding that the transferases of the Alpha multigene family are the most important contributors in the GSH conjugation of BI [selective towards the (*S*)-enantiomer] whereas the isoenzymes of the Mu family [selective towards the (*R*)-enantiomer] are the predominant enzymes in the conjugation of BIU.

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