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GLUTATHIONE CONJUGATION OF THE FLUOROPHOTOMETRIC EPOXIDE SUBSTRATE, 7-GLYCIDOXYCOUMARIN (GOC), BY RAT LIVER GLUTATHIONE TRANSFERASE ISOENZYMES

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Abstract—The fluorophotometric substrate, 7-glycidoxycoumarin (GOC), was examined for the assay of epoxide–glutathione (GSH)-conjugating activities of seven major GSH transferases (GSTs) isolated from rat liver cytosols. GST 7-7 (GST-P), isolated from the liver cytosol of rats bearing hepatic hyperplastic nodules, catalysed the GSH conjugation of GOC at a higher rate than any other examined GST isolated from the normal rat liver cytosol. GSTs 3–3, 3–4 and 4–4 (group 3–4 enzymes) had specific activities towards GOC by one fifth to one third of that of GST 7–7. GSTs 1–1, 1–2 and 2–2 (group 1–2 enzymes) had very low activities towards this epoxide. A kinetic study indicated that GST 7–7 showed the largest k_{cat}/K_m value for the catalytic reaction of GOC–GSH conjugation among the GSTs. In spite of their much smaller k_{cat} values, group 3–4 enzymes showed much larger k_{cat}/K_m values for GOC than the group 1–2 enzymes, because GOC had a much higher affinity for group 3–4 enzymes than for group 1–2 enzymes. A comparative study was also done with GSH conjugations of styrene 7,8-oxide (STO) and 1-chloro-2,4-dinitrobenzene by the GSTs. Unlike GOC, the conjugation of STO was mediated at rates about twice as high by group 3–4 enzymes than by GST 7–7. STO was also a very poor substrate for group 1–2 enzymes.

The reactive electrophiles, epoxides (oxirans), are the most representative ultimate forms of metabolites of olefinic and arylic carcinogens [1, 2]. Epoxides are enzymically detoxified mainly by glutathione (GSH) transferases (GSTs) [3] in the presence of GSH and/or by epoxide hydrolases (EHs) [2, 4, 5].

7-Glycidoxycoumarin (GOC: 7-(2′,3′-epoxy)-propoxycoumarin) has been proposed by Watabe *et al.* as a new fluorophotometric epoxide substrate for the simultaneous assay of GST [6] and EH [7] activities of various organs and tissues of animal. GOC has a 7-oxygenated coumarin structure, a well-known chromophor for the intense fluorescence emission, and because of its considerable stability, is easy to prepare, store and handle compared with other already proposed sensitive substrates, such as radioactive styrene 7,8-oxide (STO) [8] with high volatility and K-region arene oxides [3, 9, 10] with very low stability.

Unlike STO [11] and the K-region oxides [10], whose hydration to glycols or rearrangement to phenols are inevitable at weakly acidic pH, GOC could be used at pH 6.5 with little formation of the non-enzymic hydration product, 7-(2′,3′-dihydroxy)-propoxycoumarin (DHC), so that non-enzymic reaction of this epoxide with GSH and the sulphydryl or other nucleophilic groups of proteins could be retarded to a negligible extent [6]. This considerably simplified the overall procedure for the enzyme assay using GOC and increased accuracy of the assay data [6]. However, nothing is known about which isoenzymes of GST are involved in the GSH conjugation of GOC in the rat liver cytosol. The present paper deals with a comparative study of GSH conjugations of GOC, [^{3}H]STO and 1-chloro-2,4-dinitrobenzene (CDNB) by seven purified isoenzymes of rat liver cytosolic GST.

MATERIALS AND METHODS

Materials. GSH, free from its oxidized form, was donated by Yamanouchi Pharmaceutical Co. Ltd (Tokyo, Japan). CDNB was purchased from Wako Pure Chemical Industries Ltd, (Osaka, Japan), and [^{3}H]STO ([7-^{3}H]styrene 7,8-oxide, 209 mCi/mmol) from the Radiochemical Centre (Amersham, Bucks, U.K.). [^{3}H]STO was diluted with freshly redistilled STO (Wako Pure Chemical Industries Ltd) and purified by HPLC carried out with monitoring the chromatogram at 254 nm on a silica gel column (μPorasil, 10 μM particle size, 3.9 × 300 mm) in isopropanol-*n*-hexane (10:90, v/v, 1.0 ml/min). The radioactive STO eluted at 6.0 min from the HPLC column had a specific radioactivity of 20 mCi/mmol and a radiochemical purity higher than 99%, based on the radioactivity to absorbancy at 254 nm.

GOC was synthesized by the previously reported method [6]. Other chemicals used were of reagent grade.

GSTs. GSTs 1–1, 1–2, 2–2, 3–3, 3–4 and 4–4 were isolated from the liver cytosol of the male normal Sprague–Dawley (SD) rats by the method of Mannervik and Jensson [12], and GST 7–7 (originally

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named GST-P) from the liver cytosol of male SD rats bearing hepatic hyperplastic nodules by the method of Satoh *et al.* [13]. Homogeneity of these purified GST preparations was confirmed by two-dimensional gel electrophoresis (isoelectric focusing/sodium dodecyl sulphate electrophoresis) as well as by the double immunodiffusion test as previously reported [12–14].

Enzyme assay. For obtaining specific activities of GSTs towards the epoxides, GOC and [^{3}H]STO, appropriate amounts of the isoenzyme (see the legend of Table 1) were preincubated at 37° for 5 min in 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 6.5 (0.9 ml) containing GSH (4 μmol), mixed with a solution (50 μl) of GOC (0.2 μmol) in methanol containing 0.5% (w/v) Tween 80 or with an acetone solution (50 μl) of [^{3}H]STO (0.2 μmol), and then incubated under the same conditions for 10–20 min. Air-tight, ground glass-stoppered 10 ml test tubes were used for the incubation to avoid the loss of the volatile epoxide [^{3}H]STO. After the incubation, the test tubes were immediately chilled in an ice bath, and the unreacted substrates were immediately removed in the presence of sodium chloride (0.5 g) by vigorous agitation with 2-butanol (2 ml), presaturated with 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 6.5, containing a saturating amount of sodium chloride, for GOC and with ethyl acetate (2 ml), presaturated with the phosphate buffer–sodium chloride solution, for [^{3}H]STO. After removal by centrifugation and aspiration of the organic phase together with coagulated enzyme protein, floating between both layers, the residual aqueous phase was re-extracted with the same organic solvents. The twice-extracted aqueous phase was diluted with an appropriate volume of 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 6.5, and directly subjected to fluorophotometry to determine the GOC–GSH conjugate as previously reported [6] or to liquid scintillation counting to determine the [^{3}H]STO–GSH conjugates. Control blank runs were carried out by using boiled enzymes under the aforementioned conditions. Non-enzymic binding of the epoxides to 4 mM GSH occurred under the above incubation conditions at the following rates: ≤ 0.01 nmol/min and 0.26 nmol/min for GOC and [^{3}H]STO. Under the same conditions, the epoxides bound to the enzyme proteins to a negligible extent.

Kinetic parameters, K_m and k_{cat}, for the GSH conjugation of GOC by GSTs were estimated by double-reciprocal plots of the substrate concentrations of 0.01–1.0 mM vs apparent reaction rates obtained in the zero-order kinetic region.

Enzymic GSH conjugation of CDNB was determined by the previously reported method [15].

Fluorophotometry, absorptiometry and radioactivity counting. Fluorophotometry of the GOC–GSH conjugate was carried out with a Hitachi model 650-40 spectrophotometer. Absorptiometry of the GSH conjugation of CDNB was carried out with a Hitachi model U-3200 double beam spectrophotometer. The radioactivity of the [^{3}H]STO–GSH conjugate was determined in a dioxan scintillator with an Aloka model 903 liquid scintillation counter.

RESULTS AND DISCUSSION

A comparative study on GSH conjugations of the epoxide substrates, GOC and STO, and of CDNB, the substrate most frequently used in studies of GSTs, was carried out by using seven major GST isoenzymes, of which six isoenzymes, GSTs 1–1, 1–2, 2–2, 3–3, 3–4 and 4–4 were isolated from the normal SD rat liver cytosol, and the isoenzyme GST 7–7 was from the liver cytosol of the animals bearing hepatic hyperplastic nodules induced by hepatocarcinogens. The GST isoenzymes used were all pure dimeric proteins, determined by two-dimensional gel electrophoresis (isoelectric focusing/sodium dodecyl sulphate electrophoresis) as well as by the double immuno-diffusion test.

The result of the comparative study indicated that of the seven isoenzymes used, GST 7–7 utilized GOC the best (Table 1). The 3–4 group enzymes, GSTs 3–3, 3–4 and 4–4, had 22–34% of the activity of GST 7-7, and the 1-2 group enzymes, GSTs 1-1, 1-2 and 2-2, showed much less activity towards GOC than the 3-4 group enzymes. In contrast to GOC, STO was a good substrate for the 3–4 group enzymes, especially for GST 3-4. Towards STO, GST 7-7 showed only 41% of the activity shown by GST 3-4. The 1-2 group enzymes also showed less activity towards STO than towards GOC. As has been demonstrated [15], CDNB was a very good substrate for all of the GST isoenzymes with a lesser difference in catalytic activity.

A kinetic study of the GOC–GSH conjugation reaction by the GST isoenzymes indicated that 3-4 group enzymes had higher affinities for GOC than the other groups of GST according to estimation by K_m values, although the k_{cat} values for these GSTs were the smallest among the three groups of enzymes (Table 2). In spite of their considerably large k_{cat} values, GSTs 1-2 and 2-2 had the largest K_m values towards GOC among the GSTs used, so that the apparent reaction rates with GSTs 1-2 and 2-2 might be much smaller than those with the other groups of enzymes (Table 1) as readily estimated by the k_{cat}/K_m values (Table 2). GST 7-7 showed the highest k_{cat}/K_m value towards GOC of all the GST isoenzymes used (Table 2). This could be reasonably attributed to GST 7-7 having the largest apparent rate in GOC–GSH conjugation (Table 1).

Various epoxides have been examined for their susceptibility to GSH conjugation by GST isoenzymes, and the results indicate that, unlike CDNB, their conjugations are catalysed at largely different rates by the isoenzymes [3, 15–18]. Conjugation of aflatoxin B_1 8,9-oxide, an ultimate form of the hepatocarcinogen aflatoxin B_1, is catalysed most effectively by GST 1-1 and at smaller rates by GSTs 1-2 and 2-2, but not by GSTs 3-3, 3-4, 4-4 and 5-5 [17]. GSH conjugation of cholesterol 5,6α-epoxide, first demonstrated by Watabe *et al.* [19], is also catalysed by 1-2 group enzymes [20]. Cholesterol 5,6α-epoxide is formed during lipid peroxidation of microsomes in the liver of various animals [21, 22] and suspected to be closely related to human colon cancer [23].

The 3-4 group enzymes play an important role in GSH conjugation not only of K-region epoxides of carcinogenic polycyclic aromatic hydrocarbons (PAHs) and azaarenes [3, 24, 25] and bay region diol-epoxides of benzo[*a*]pyrene (BaP) [26], but also of the methyl ester of leukotriene A_4 (LTA_4 Me) to

Table 1. A comparative study of GSH conjugation of 7-glycidoxycoumarin (GOC), styrene 7,8-oxide (STO) and 1-chloro-2,4-dinitrobenzene (CDNB) by major GSTs of normal and hepatic hyperplastic nodule-bearing SD rat liver cytosols

GST	Enzyme activities (% relative activities) GOC (nmol/mg protein/min)	STO (nmol/mg protein/min)	CDNB (μmol/mg protein/min)
1-1 (Ya-Ya)	6.3 (4.1)	3.3 (2.5)	33.0 (100)
1-2 (Ya-Yc)	7.4 (4.8)	3.6 (2.7)	26.0 (78.7)
2-2 (Yc-Yc)	11.1 (7.1)	3.3 (2.5)	17.2 (52.1)
3-3 (Yb_1-Yb_1)	35.5 (22.9)	133.5 (99.8)	28.3 (85.7)
3-4 (Yb_1-Yb_2)	53.0 (34.1)	133.8 (100)	32.9 (99.6)
4-4 (Yb_2-Yb_2)	45.1 (29.0)	104.7 (78.3)	19.6 (59.4)
7-7 (Yp-Yp)	155.5 (100)	55.3 (41.3)	15.2 (46.1)

Concentrations of the substrates used were 0.2, 0.2 and 1 mM for GOC, STO and CDNB, respectively. GOC was incubated at pH 6.5 with GSTs 1-1, 1-2 and 2-2 (1.6 μg protein/ml), GSTs 3-3, 3-4 and 4-4 (0.3 μg protein/ml) and GST 7-7 (0.1 μg protein/ml), STO at pH 6.5 with GSTs 1-1, 1-2 and 2-2 (10 μg protein/ml) and with the other GSTs (3 μg protein/ml), and CDNB at pH 8.0 with the GSTs (0.8 μg protein/ml).

Table 2. Kinetic parameters for GSH conjugation of GOC by rat liver cytosolic GSTs

GST	GOC k_{cat}* (/sec)	K_m (mM)	k_{cat}/K_m (/mM/sec)
1-1	0.06	2.0	0.03
1-2	0.10	2.5	0.04
2-2	0.30	3.0	0.10
3-3	0.02	0.12	0.17
3-4	0.04	0.11	0.36
4-4	0.03	0.08	0.38
7-7	0.53	0.58	0.91

* Molecular weights of GSTs are based on Ketterer *et al.* [46].

LTC_4 Me [27]. As to BaP-7,8-diol 9,10-epoxides, GSTs 3-4 and 4-4 have much higher activities than does GST 3-3, while as to the K-region epoxides, GST 3-3 has an activity comparable to GSTs 3-4 and 4-4 [24–26]. These GST isoenzymes, therefore, are considered to play a major part in detoxifying the epoxides as ultimate metabolites of carcinogenic PAHs in the rat liver. Even for such a small carcinogenic epoxide molecule as STO [28, 29], the present study indicates that 3-4 group enzymes play an important role in its detoxication (Table 1). Regarding rat liver GSTs, STO has been reported to be a good substrate for GST 5-5 which is a very minor GST component, approximately 0.2% of total cytosolic GST proteins in the Wistar rat liver, in contrast to the high contents of group 3-4 enzymes in the same liver cytosol, 44% of total GSTs: made up of 13, 25 and 6% for GSTs 3-3, 3-4 and 4-4, respectively [30]. In the human liver cytosol, however, GSH conjugation of STO has been demonstrated to be catalysed by the GST isoenzymes in the order $\mu > \pi > \alpha \sim \varepsilon$ [31, 32]. These results are of interest in view of the previously reported homology of the rat liver GST isoenzymes to those of the human liver; group 1-2 and 3-4 enzymes and GST 7-7 of rat liver have very similar physico- and immunochemical properties and substrate specificity to those of human liver GSTs $\alpha \sim \varepsilon$, μ and π, respectively [33].

GST 7-7, having the highest activity towards GOC among the GST isoenzymes used, does not exist at a detectable level in the normal rat liver cytosol [13, 34–36], but is markedly induced at the highest level, comparable to that of the inducible isoenzyme GST 3-3, among the GST isoenzymes in the liver of rats bearing hepatic hyperplastic nodules induced by hepatocarcinogens [13, 34, 36, 37]. Consequently, GST 7-7 has been considered as a marker protein for carcinogen-induced primary hepatoma in the rat [13, 34, 37, 38]. The isoenzyme also exists, although generally in low quantities, in normal tissues such as placenta [13], liver [13], small intestine [39], brain [40] and skeletal muscle [16] except that the level is significantly higher in kidney [13, 41] or lung [13, 42], but is still much less than that in the preneoplastic tissues. GST 7-7 has been demonstrated to play an important role, together with GSTs 3-4 and 4-4, in detoxifying BaP-7,8-diol 9,10-epoxides [43], and synthesizing LTC_4 from LTA_4 [40]. Ethacrynic acid has been used as a standard substrate showing the highest selectivity towards GST 7-7 of the rat liver GST isoenzymes [16], except for GST 8-8, a very minor GST component [44, 45]. However, based on our experience, it should be emphasized that, from the viewpoint of sensitivity, GOC was a more useful substrate than the absorptiometric substrate, ethacrynic acid, for the assay of GST 7-7.

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