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Molecular and Catalytic Properties of Glutathione Transferase μ from Human Liver: An Enzyme Efficiently Conjugating Epoxides†

Margareta Warholm, Claes Guthenberg, and Bengt Mannervik*

ABSTRACT: Glutathione transferases with basic isoelectric points are present in the cytosol of all human livers investigated. Adults as well as fetuses contain such basic proteins, referred to as transferases α–ϵ. Some adult (about 60%), but no fetal, livers have a different enzyme, glutathione transferase μ, with an isoelectric point at pH 6.6 [Warholm, M., Guthenberg, C., Mannervik, B., & von Bahr, C. (1981) *Biochem. Biophys. Res. Commun. 98*, 512–519]. This enzyme has also been found in adult adrenal glands. Chemical and physical properties distinguish three major types of human transferases: transferase μ, transferases α–ϵ, and transferase π from human placenta. Transferase μ has two subunits of M_r 26 300; M_r 53 000 and Stokes radius = 3.0 nm were determined independently for the native dimeric protein. Analyses of the amino acid compositions show that the three types of transferases are not interconvertible by posttranslational modifications. Antibodies against any of the human transferases did not cross-react with the other proteins or with rat liver glutathione transferases. Circular dichroism spectra in the near-ultraviolet region are clearly different for the three types of transferases. Estimation of the secondary protein structure from the circular dichroism in the far-ultraviolet region gave 23% α-helix and 25% β-structure for transferase μ. Noteworthy kinetic properties of transferase μ are high specific activities with *trans*-4-phenyl-3-buten-2-one, benzo[*a*]pyrene 4,5-oxide, and styrene 7,8-oxide; pH optimum at 7.5. A random order sequential reaction scheme could explain the steady-state kinetics; experimental data were evaluated by means of nonlinear regression analysis. Transferase μ is highly efficient with benzo[*a*]pyrene 4,5-oxide as substrate: K_m = 0.9 μM, and $k_{cat}/K_m = 3.2 \times 10^7$ min^{-1} M^{-1}. Glutathione derivatives with hydrophobic S-substituents were strong reversible inhibitors: K_i = 0.75 μM (competitive with glutathione) for *S*-*n*-hexylglutathione. Deoxycholate (K_i = 21 μM) and cholate (K_i = 40 μM) were both competitive with the second substrate, 1-chloro-2,4-dinitrobenzene, whereas bromosulfophthalein (K_i = 0.8 μM) and bilirubin (nonlinear inhibition) were noncompetitive with both substrates. Equilibrium binding measured by the quenching of the intrinsic protein fluorescence indicated hyperbolic saturation with bromosulfophthalein (K_d = 1 μM) and bilirubin ($K_d \sim 10$ μM). Transferase μ was irreversibly inactivated by Hg^{2+}, *N*-ethylmaleimide, *N*-phenylmaleimide, 2,4,6-trinitrobenzenesulfonate, and 1-fluoro-2,4-dinitrobenzene. The results indicate strongly that three genetically distinct types of human glutathione transferases exist. The high activity of transferase μ with epoxides may provide better protection against some chemical mutagens and carcinogens to those individuals having transferase μ in their tissues.

The glutathione transferases (EC 2.5.1.18) are a group of multifunctional proteins assumed to be important in the detoxification of many different endogenous and exogenous compounds (Jakoby & Habig, 1980). The most extensive studies of the different forms of the transferases have been performed in rat liver (Jakoby & Habig, 1980), but recently, information about these enzymes in human tissues has also started to appear. Five very similar proteins with basic isoelectric points, referred to as transferases α, β, γ, δ, and ϵ, were first isolated from human liver (Kamisaka et al., 1975). Later, Awasthi et al. (1980) and Koskelo & Valmet (1980) reported the presence of glutathione transferases with acidic isoelectric points in human liver. Similar or identical acidic glutathione transferases have been purified from erythrocytes (Marcus et al., 1978), placenta (Guthenberg et al., 1979; Guthenberg & Mannervik, 1981), and lung (Koskelo et al., 1981; Mannervik et al., 1983). We have shown that an additional form of glutathione transferase exists in the liver of some individuals (Warholm et al., 1980). This form, transferase μ, with a near-neutral isoelectric point, has been purified to apparent homogeneity (Warholm et al., 1981a). We now

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report some molecular and catalytic properties of the purified glutathione transferase μ. The results clearly show that transferase μ is a distinct enzyme form, separate from the human transferases previously characterized, and most likely the product of a gene that is different from those of the other transferases.

Experimental Procedures

Materials. Benzo[*a*]pyrene 4,5-oxide was a gift from the Cancer Research Program of the National Cancer Institute, Division of Cancer Cause and Prevention, Bethesda, MD. Styrene 7,8-oxide was obtained from Dr. Joseph W. DePierre of the Department of Biochemistry, University of Stockholm, Stockholm. All other chemicals were standard commercial products.

Preparation of Enzyme. Glutathione transferase μ was purified to homogeneity from human liver, obtained from kidney donors, as previously described (Warholm et al., 1980, 1981a).

Antibodies. Antibodies to transferase μ, the hepatic basic transferases, and transferase π from human placenta were raised in rabbits.

Methods. The molecular weight was determined by a combination of gel filtration and ultracentrifugation (Siegel & Monty, 1966). Reference proteins (with their respective Stokes radii in parenthesis) were lactate dehydrogenase (4.1 nm), bovine serum albumin (3.55 nm), glyoxalase I (2.8 nm), and cytochrome *c* (1.64 nm). For the estimation of the sedimentation coefficient, catalase ($s_{20,w}$ = 11.3 S) was used as a reference. The subunit molecular weight was estimated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Laemmli, 1970) with bovine serum albumin (M_r 67 000), carbonic anhydrase (M_r 29 000), human erythrocyte glyoxalase I (subunit M_r 23 000), and ribonuclease (M_r 13 800) as standards. Protein concentration was determined by the method of Lowry et al. (1951).

Amino acid analyses were carried out by Dr. D. Eaker, Institute of Biochemistry, University of Uppsala, Uppsala. Protein samples, dialyzed against 10 mM ammonium acetate, were hydrolyzed in 6 M HCl for 24 and 72 h and analyzed on a Durrum D-500 amino acid analyzer with norleucine as an internal standard. Tryptophan was determined separately (Penke et al., 1974).

The number of thiol groups were estimated by Ellman's procedure (1959) after denaturation of the protein in 6 M guanidinium chloride. The isoelectric point was determined by isoelectric focusing at 4 °C in a sucrose gradient with Ampholine, pH 5–8 (LKB Produkter, Stockholm), as the ampholytes.

Circular dichroism spectra were run in 0.1 M sodium phosphate buffer (pH 6.5) on a Jasco J-41A spectropolarimeter. Rectangular cells with thicknesses ranging from 0.25 to 10 mm were used. The measurements are expressed as mean residue ellipticity, $[\theta]_{\bar{M}_r}$, with a mean residue weight ($\bar{M}_r$) of 117 for glutathione transferase μ.

Kinetic Studies. Glutathione transferase activities with various substrates were measured as described by Warholm et al. (1981a). Catalytic activity with styrene 7,8-oxide was determined as described by DePierre & Moron (1979). All kinetic experiments were performed at 30 °C. Reactions were started by addition of enzyme.

The effect of reversible inhibitors on the steady-state kinetics was studied in 0.1 M sodium phosphate (pH 6.5) by use of 1-chloro-2,4-dinitrobenzene and glutathione as substrates.

The kinetic data were analyzed by use of weighted nonlinear regression analysis (Mannervik, 1982).

Table I: Amino Acid Composition of Human Glutathione Transferases[a]

amino acid	transferase μ	basic transferases	transferase π
Asx	50.4	37.6	42.1
Thr	13.4	8.5	18.4
Ser	21.4	25.0	20.2
Glx	49.9	51.9	49.2
Pro	20.2	24.3	23.6
Gly	30.0	22.2	37.4
Ala	22.8	31.5	32.0
Cys	9.2	2.0	8.0
Val	12.6	19.3	28.1
Met	12.4	14.9	4.8
Ile	27.0	29.8	13.5
Leu	55.6	57.7	62.4
Tyr	24.0	20.6	24.0
Phe	26.0	19.9	13.8
His	11.0	5.8	3.9
Lys	40.0	47.4	24.2
Trp	6.8	3.2	3.4
Arg	19.8	23.3	15.9

[a] The compositions were calculated by using M_r 53 000, 51 000, and 47 000 for transferase μ, the basic transferases (α–ε), and transferase π, respectively.

The inactivation of transferase μ with group specific reagents was carried out in a separate incubation system. The system contained 0.1 M sodium phosphate (pH 8.0), a suitable amount of enzyme, and inactivator at a concentration of 1.3 mM in a final volume of 87 μL. After incubation at 25 °C for 20 min, aliquots (20 μL) of the incubation system were transferred to the test system for determination of the residual catalytic activity.

Binding Studies. The dissociation constants for the binding of bilirubin and bromosulfophthalein to transferase μ were determined by analysis of the quenching of the intrinsic protein fluorescence upon titration of the protein with the ligand. Measurements were performed at 22 °C in 0.1 M sodium phosphate (pH 6.5) on a Jasco FP-4 spectrofluorometer: λ_{ex} = 280 nm, and λ_{em} = 335 nm. The fluorescence values were corrected for inner filter effects (McClure & Edelman, 1967). Evaluation of the titration curves was made by nonlinear regression analysis as earlier described (Aronsson et al., 1981). A 1 mM stock solution of bilirubin in 10 mM NaOH was prepared, stored in darkness on ice, and used within 3 h.

Results

Molecular Properties. The molecular weight of glutathione transferase μ purified from human liver was estimated as 53 000. The Stokes radius was 3.0 nm. In the presence of sodium dodecyl sulfate, the protein dissociated into two apparently identical subunits of M_r 26 300, as determined by polyacrylamide gel electrophoresis. These results as well as the results of parallel runs with the basic and the acidic transferases show that transferase μ is a slightly larger protein than the other human transferases (Kamisaka et al., 1975; Guthenberg & Mannervik, 1981). The isoelectric point, determined by isoelectric focusing at 4 °C in a pH gradient of 5–8, was at pH 6.6.

Amino Acid Composition. The amino acid composition of glutathione transferase μ was determined in two samples originating from separate purifications of the enzyme. The values in Table I are the means of the two analyses. The amino acid compositions of transferase π and the hepatic basic transferases, respectively, are listed for comparison. As a quantitative measure of similarity between the amino acid compositions of the various human transferases, the difference

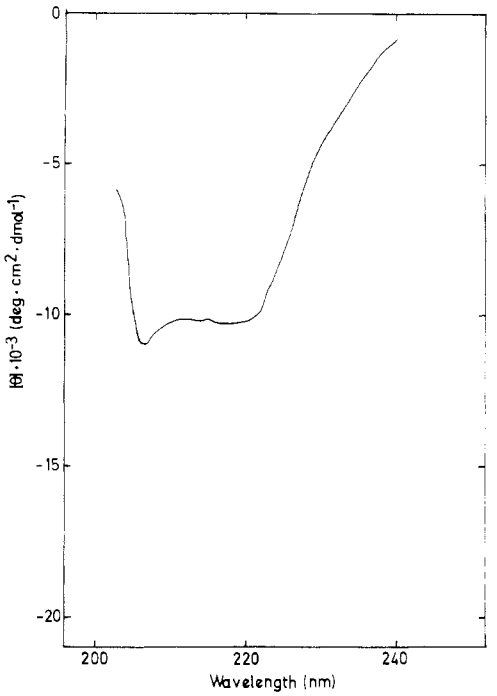

FIGURE 1: Circular dichroism of glutathione transferase μ in the far-ultraviolet region. The spectrum was recorded in 0.1 M sodium phosphate (pH 6.5).

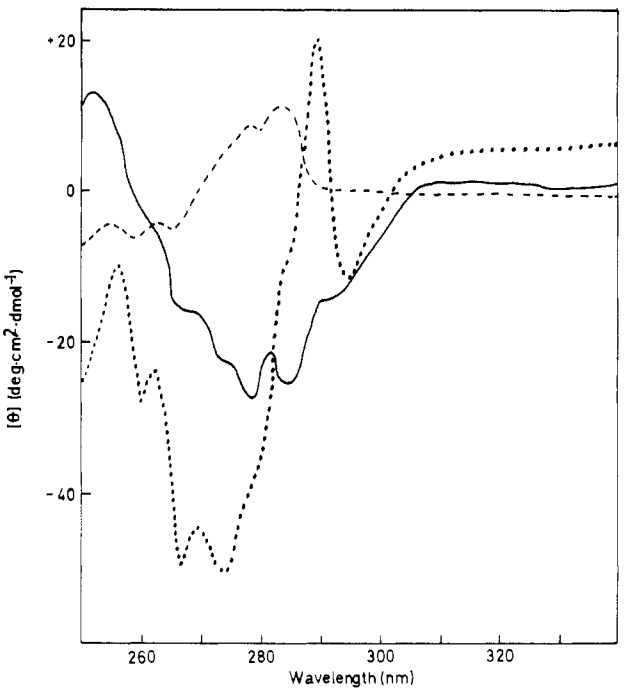

FIGURE 2: Circular dichroism of human glutathione transferases in the near-ultraviolet region. The spectra were recorded in 0.1 M sodium phosphate (pH 6.5): (---) transferase μ, (—) transferase π from human placenta, and (···) basic glutathione transferases from human liver.

index of Metzger et al. (1968) was used. A value of 11 was obtained when transferase μ and the basic forms were compared. Corresponding values for transferase μ vs. transferase π and for transferase π vs. the basic transferases were 14 and 15, respectively.

For comparison, the difference index for transferase "A" vs. transferase "C" from rat liver has been reported to be 2.2 (Habig et al., 1974). This value is not unbiased (and probably too low), because one of the subunits of transferase C (now called transferase AC) is identical with the subunits of transferase A (now transferase A_2) (Mannervik & Jensson, 1982). Nevertheless, the significantly larger difference indexes for the human transferases indicate that the three types of human glutathione transferases have distinctly different primary structures.

The total number of titratable sulfhydryl groups of transferase μ was found to be 5.7, as measured with 5,5′-dithiobis(2-nitrobenzoate) after denaturation of the protein in 6 M guanidinium chloride.

Circular Dichroism. The circular dichroism spectrum of transferase μ in the far-ultraviolet region is shown in Figure 1. Analysis of the secondary structure was made as described by Chen et al. (1972). The computed estimates were 23% α-helix and 25% β-structure. Figure 2 shows the spectrum in the near-ultraviolet region. The spectrum of transferase μ is completely different from the corresponding spectra of transferase π and the basic transferases (Figure 2), indicating major structural differences.

Immunochemical Properties. Antibodies were raised in rabbits to transferase μ, to the hepatic basic forms, and to transferase π from human placenta, respectively. Each antiserum reacted with its corresponding antigen but not with the other transferases, as judged from results of Ouchterlony double-diffusion experiments. Thus, transferase μ does not cross-react immunologically with the other human transferases. Similar experiments with antibodies to glutathione transferases from rat liver cytosol (Guthenberg et al., 1980) show no reaction with human transferase μ, nor do antibodies against transferase μ react with the rat liver enzymes.

Table II: Substrate Specificities of Glutathione Transferases

substrate	specific activity (μmol min^{-1} mg^{-1}) for transferases: μ	basic (α-ϵ)	π[a]
1-chloro-2,4-dinitrobenzene	187	64	105
1,2-dichloro-4-nitrobenzene	0.032	0.035–0.065[b]	0.11
bromosulfophthalein	<0.002	0.001–0.01[b]	<0.002
ethacrynic acid	0.081	0.017–0.044[b]	0.86
trans-4-phenyl-3-buten-2-one	0.36	0.001–0.002[b]	0.01
1,2-epoxy-3-(*p*-nitrophenoxy)-propane	0.11	0[b]	0.37
styrene 7,8-oxide	2.6	0.02	0.14
benzo[*a*]pyrene 4,5-oxide	0.92	0.047	0.13
cumene hydroperoxide	0.63	10.6	0.03
Δ^5-androstene-3,17-dione	0.12	8.0	0.01
p-nitrophenyl acetate	0.22	0.18	0.19

[a] Data reported by Guthenberg & Mannervik (1981). [b] Data reported by Jakoby & Habig (1980).

Kinetic Properties. The substrate specificities of transferase μ, the hepatic basic transferases, and transferase π from placenta are shown in Table II. Transferase μ has comparatively high activities with 1-chloro-2,4-dinitrobenzene, *trans*-4-phenyl-3-buten-2-one, benzo[*a*]pyrene 4,5-oxide, and styrene 7,8-oxide. The pH optimum for the enzymatic reaction between glutathione and 1-chloro-2,4-dinitrobenzene was at approximately pH 7.5. The same pH optimum was obtained for the conjugation of glutathione with *trans*-4-phenyl-3-buten-2-one (Figure 3).

The steady-state kinetics of the reactions between glutathione and 1-chloro-2,4-dinitrobenzene or *trans*-4-phenyl-3-buten-2-one, respectively, were studied at pH 6.5 and could be approximated by the rate equation for a simple random sequential mechanism. The kinetic constants, estimated by

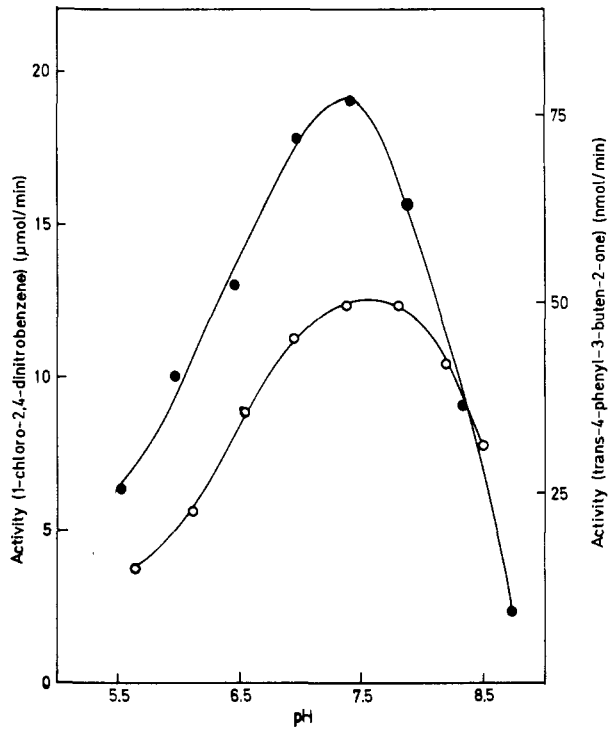

FIGURE 3: pH dependence of initial velocities obtained with two different substrates in universal buffer (Britton & Welford, 1937). The experimental details are described under Experimental Procedures. (●) 1-Chloro-2,4-dinitrobenzene; (○) *trans*-4-phenyl-3-buten-2-one.

Table III: Kinetic Constants for Different Substrates of Glutathione Transferase μ[a]

parameter	value ± SD
1-chloro-2,4-dinitrobenzene[b]	
k_{cat} (min^{-1})	11600 ± 1500
K_m^A(glutathione) (mM)	0.16 ± 0.05
K_m^B(1-chloro-2,4-dinitrobenzene) (mM)	0.65 ± 0.11
K (mM^2)	0.22 ± 0.03
trans-4-phenyl-3-buten-2-one[c]	
k_{cat} (min^{-1})	121 ± 69
K_m^A(glutathione) (mM)	0.81 ± 0.61
K_m^B(*trans*-4-phenyl-3-buten-2-one) (mM)	0.33 ± 0.22
K (mM^2)	0.079 ± 0.040
benzo[*a*]pyrene 4,5-oxide[d]	
k_{cat} (min^{-1})	28.8 ± 1.3
K_m(benzo[*a*]pyrene 4,5-oxide) (μM)	0.90 ± 0.15

[a] The kinetic data were analyzed by weighted nonlinear regression analysis by using the equation $v/[E]_{tot} = k_{cat}[A][B]/(K_m^B[A] + K_m^A[B] + [A][B] + K)$ and a weighting function: $w_i \propto v_i^{-2}$ (cf. Mannervik, 1982). [b] Concentrations of 1-chloro-2,4-dinitrobenzene and glutathione varied independently in the ranges 1–1000 and 1–5000 μM, respectively. Two independent data sets (n = 43 and 51, respectively) were fitted simultaneously in order to allow for "interexperiment" variance (cf. Mannervik, 1982). [c] Concentrations of *trans*-4-phenyl-3-buten-2-one and glutathione varied independently in the ranges 10–200 μM and 0.1–1.0 mM, respectively (n = 20). A cuvette with a 2-mm light path was used. [d] The concentration of benzo[*a*]pyrene 4,5-oxide varied in the range 0.6–10 μM at a constant (physiological) concentration of 5 mM glutathione (n = 5).

weighted nonlinear regression analysis, are given in Table III. A kinetic study limited to one concentration of glutathione (5 mM) was also made with benzo[*a*]pyrene 4,5-oxide at pH 7.7. The K_m for benzo[*a*]pyrene 4,5-oxide was estimated as 0.9 μM, which is at least 2 orders of magnitude lower than for the other two substrates.

Inhibition Studies. The inhibitory effect of various glutathione derivatives was studied. Table IV shows that the strongest inhibition was obtained with the most hydrophobic

Table IV: Inhibition of Glutathione Transferase μ with S-Substituted Glutathione Derivatives[a]

inhibitor	inhibition (%)
glutathione disulfide	<2
S-methylglutathione	14
S-*n*-hexylglutathione	91
S-*n*-octylglutathione	97
S-(*p*-bromobenzyl)glutathione	98
S-(2-chloro-4-nitrophenyl)glutathione	68
S-(bromosulfophthaleinyl)glutathione	94

[a] The inhibitory effect of the compounds was tested at 0.1 mM concentration in an assay system containing 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene in 0.1 M sodium phosphate (pH 6.5). The reaction was started by addition of enzyme.

Table V: Apparent Inhibition Constants for Some Linear Inhibitors of Glutathione Transferase μ[a]

inhibitor	apparent constant ± SD (μM)
bromosulfophthalein[b]	K_{ii} = 0.88 ± 0.10
	K_{is} = 0.83 ± 0.05
S-*n*-hexylglutathione[c]	K_{is} = 0.75 ± 0.05
cholate[d]	K_{is} = 40.2 ± 1.6
deoxycholate[d]	K_{is} = 21.2 ± 1.6

[a] Kinetic data sets were analyzed by weighted nonlinear regression analysis by using the equation $v = V[A]/(K_m + [A] + K_m[I]/K_{is} + [A][I]/K_{ii})$ where K_{is} and K_{ii} are inhibition constants and [A] and [I] the varied substrate and inhibitor concentrations. For competitive inhibition, $K_{ii} \to \infty$, the last term of the denominator was omitted in computing the parameter values. Weighting factors were $w_i \propto v_i^{-2}$. [b] Noncompetitive inhibition with 1-chloro-2,4-dinitrobenzene concentration varied (at 1 mM glutathione). Similar results were obtained with glutathione concentration varied (at 0.5 mM 1-chloro-2,4-dinitrobenzene). [c] Competitive inhibition with glutathione concentration varied (at 0.5 mM 1-chloro-2,4-dinitrobenzene). [d] Competitive inhibition with 1-chloro-2,4-dinitrobenzene (at 1 mM glutathione).

S-substituents. Glutathione disulfide was a poor inhibitor.

The inhibitory action of some compounds was studied more extensively. Apparent inhibition constants obtained by weighted regression analysis are given in Table V. Rigorously, the listed values are dependent on the concentration of the nonvaried substrate, but for numerical reasons the estimates do not differ by large measures from the true values.

Bromosulfophthalein was found to be a potent reversible inhibitor of transferase μ. The inhibition was linear with inhibitor concentration and noncompetitive toward both glutathione and 1-chloro-2,4-dinitrobenzene, with an apparent K_i of approximately 1 μM (Figure 4).

The inhibition by bilirubin was also noncompetitive with both glutathione and 1-chloro-2,4-dinitrobenzene but was more complex as shown by nonlinear graphs in Dixon plot (Figure 5). Inhibition of 50% was obtained at 2–5 μM concentration. The kinetic data could be described by a 1:2 function in inhibitor concentration ([I]):

$$v = \frac{K_1[A] + K_2[A][I]}{K_3 + [A] + K_4[I] + K_5[A][I] + K_6[I]^2}$$

where K_i (i = 1–6) are constants and [A] is the concentration of the varied substrate (1-chloro-2,4-dinitrobenzene).

S-*n*-Hexylglutathione was shown to be competitive with glutathione and noncompetitive with 1-chloro-2,4-dinitrobenzene, with a K_i of about 1 μM. Both effects were linear with respect to inhibitor concentration.

The inhibitory effects of two bile acids were also examined. Sodium deoxycholate was a slightly better inhibitor ($K_i \sim 20$

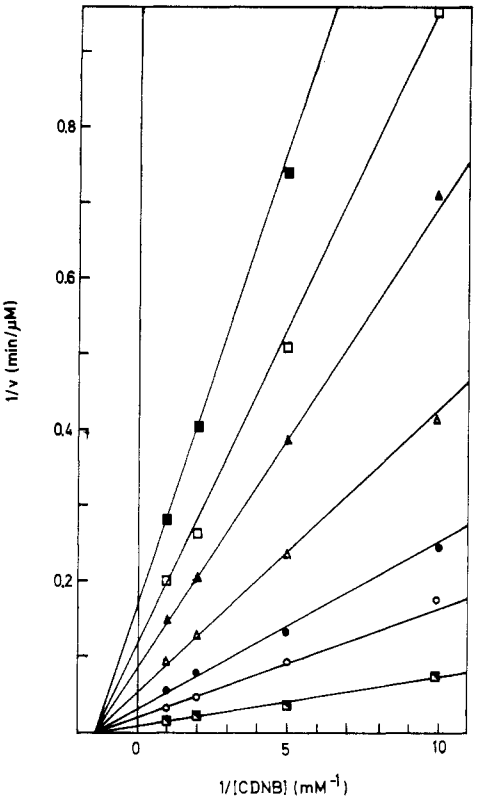

FIGURE 4: Effect on initial velocity of bromosulfophthalein with 1-chloro-2,4-dinitrobenzene as the varied substrate. The concentrations of bromosulfophthalein were (half-filled square) 0, (○) 1, (●) 2, (△) 4, (▲) 7, (□) 10, and (■) 15 μM. The concentration of glutathione was 1.0 mM; the concentration of 1-chloro-2,4-dinitrobenzene (CDNB) varied between 0.1 and 1.0 mM.

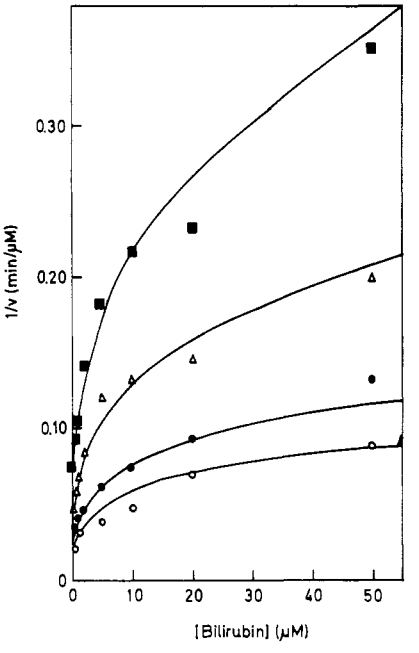

FIGURE 5: Inhibition of glutathione transferase μ with bilirubin (Dixon plot). The concentration of glutathione was 0.97 mM. Four different concentrations of 1-chloro-2,4-dinitrobenzene were used: (○) 1.0, (●) 0.5, (△) 0.2, and (■) 0.1 mM.

μM) than sodium cholate ($K_i \sim 40$ μM). Both inhibitors were competitive with 1-chloro-2,4-dinitrobenzene.

Double inhibition experiments with bromosulfophthalein and *S*-*n*-hexylglutathione did not give parallel lines in a Dixon plot (Figure 6), showing that the two inhibitors do not bind at exactly the same site. Sodium cholate and sodium deoxycholate, on the other hand, showed exclusive binding (data not shown).

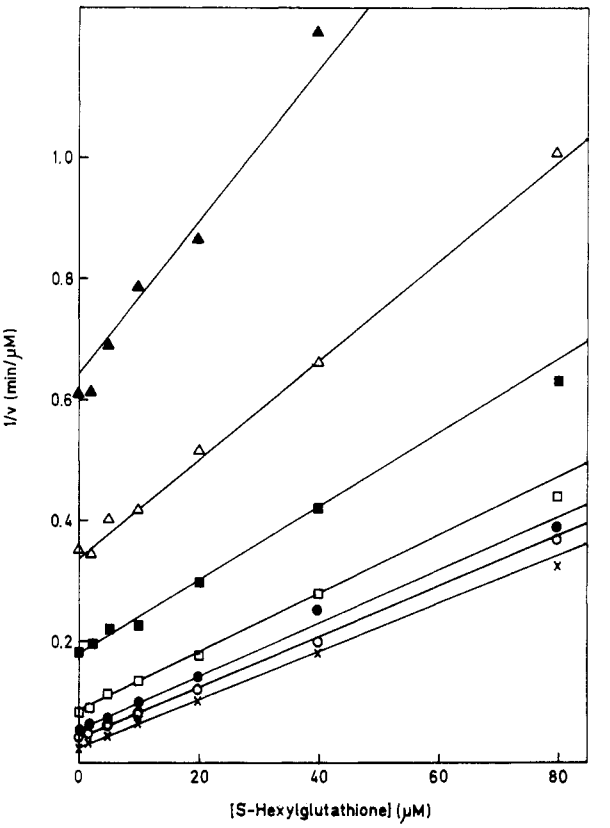

FIGURE 6: Double-inhibition experiment involving bromosulfophthalein and *S*-*n*-hexylglutathione as inhibitors. The fixed substrate concentrations were 1.1 mM glutathione and 0.5 mM 1-chloro-2,4-dinitrobenzene. Seven different concentrations of bromosulfophthalein were used: (×) 0, (○) 0.5, (●) 1.0, (□) 2.0, (■) 5.0, (△) 10, and (▲) 20 μM. The concentration of *S*-*n*-hexylglutathione varied between 0 and 80 μM.

Effect of Group-Specific Reagents. Glutathione transferase μ was inactivated extensively by incubation with both mercaptide-forming and alkylating thiol reagents. For example, Hg^{2+}, *N*-ethylmaleimide, or *N*-phenylmaleimide at 1 mM concentration gave >98% inactivation in 20 min. This finding indicates that the enzyme is dependent on thiol groups for catalytic activity. 2,4,6-Trinitrobenzenesulfonate and 1-fluoro-2,4-dinitrobenzene, both known to block amino groups, gave 90% and 97% inhibition of the catalytic activity under the same conditions. However, the latter reagents may also react with thiol groups, and definite identification of the nature of the essential amino acid residues requires further investigation.

Binding Studies. The binding of bromosulfophthalein and bilirubin to transferase μ was determined by measuring the quenching of the intrinsic fluorescence upon addition of ligand. The binding isotherms were hyperbolic for both ligands. The dissociation constant for the binding of bilirubin to transferase μ was found to be about 10 μM. Addition of 1 mM glutathione did not change the strength of binding. The binding of bromosulfophthalein was 10-fold stronger. The K_d value of bromosulfophthalein (1 μM) determined fluorometrically is in good agreement with K_i values estimated by kinetic measurements (Table V).

Discussion

Occurrence of Glutathione Transferase μ. A conspicuous finding was that glutathione transferase μ, in contrast to the basic cytosolic transferases, is present in the liver of only some

individuals. The frequency of occurrence is approximately 60% (present in 13 of the 23 livers tested) (Warholm et al., 1980). The enzyme has been found in both males and females (Warholm et al., 1980). Glutathione transferase μ has also been identified in the adrenal gland, but it has, so far, only been found in adult and not in fetal tissues (Mannervik et al., 1983).

Molecular Properties. Several lines of evidence show that glutathione transferase μ is distinct from the basic transferases α–ε (Kamisaka et al., 1975) and from transferase π (Guthenberg & Mannervik, 1981). Transferases α–ε, on the other hand, appear to be very similar in most respects (Kamisaka et al., 1975). The isoelectric point at pH 6.6 for transferase μ is different from those for transferases α–ε (pH >7.8) and transferase π (pH 4.8). The size of transferase μ (M_r 53 000) is significantly greater than the sizes of transferases α–ε (M_r 51 000) and transferase π (M_r 47 000). All the glutathione transferases are proteins composed of two subunits of similar size, and the differences in the molecular weight values of the dimeric proteins have their counterparts in differences in sizes of the subunits. The possibility that transferase μ, the largest molecule, gives rise to transferases α–ε and/or transferase π by way of limited proteolysis is excluded by the results of the amino acid analyses (Table I). For example, it is impossible that the content of valine could rise from 12.6 per molecule to 19.3 and 28.1 in a conversion of the largest protein to the smaller ones by partial proteolytic degradation. Values for other amino acids make it possible to draw the same conclusion. As a corollary it can be stated that neither can transferase π arise posttranslationally from any of transferases α–ε.

Double-immunodiffusion experiments did not reveal any cross-reactivity between the three different types of human glutathione transferases and their corresponding antibodies. This finding supports the above conclusion about the distinct differences between the transferases. Furthermore, the near-ultraviolet circular dichroism spectra of the three types of transferases (Figure 2) appear completely unrelated. The spectral region used reflects asymmetric interactions with the environment of aromatic amino acid residues, and the spectrum serves as a "fingerprint" in the discrimination between protein molecules.

Thus, the molecular properties show conclusively that the three groups of human glutathione transferases are not interconvertible by posttranslational events. The differences in substrate specificities (see below) are consistent with this conclusion.

Kinetic Properties. The steady-state kinetics of glutathione transferase μ with 1-chloro-2,4-dinitrobenzene and glutathione as substrates were evaluated in accordance with a random sequential mechanism. This simple kinetic pattern may appear different from the rate behavior previously demonstrated for glutathione transferase A_2 from rat liver [previously named transferase A; see Mannervik & Jensson (1982)] in experiments with 1,2-dichloro-4-nitrobenzene as the electrophilic substrate (Jakobson et al., 1977, 1979a). Nevertheless, for both enzymes the kinetics are consistent with the same basic reaction scheme, i.e., a ternary complex mechanism with random order addition of the substrates (Jakobson et al., 1979b).

The kinetic constants determined for human glutathione transferase μ (Table III) are similar to those determined for transferase π (Guthenberg & Mannervik, 1981). The ratio k_{cat}/K_m for 1-chloro-2,4-dinitrobenzene is 1.8×10^7 min^{-1} M^{-1}, which is high but still two decades lower than k_{cat}/K_m for the most efficient enzymes (Fersht, 1977). More important from the physiological point of view is the k_{cat}/K_m for benzo[*a*]pyrene 4,5-oxide, which is 3.2×10^7 min^{-1} M^{-1}. This is the highest ratio determined for transferase μ, so far, suggesting that the enzyme may have been designed for conjugation of arene oxides and similar substrates. The k_{cat} value for *trans*-4-phenyl-3-buten-2-one is approximately 100 times lower than that for 1-chloro-2,4-dinitrobenzene (Table III). On the basis of the specific activities, this lower k_{cat} value is more representative of all other substrates tested (Warholm et al., 1981a).

The specific activities with benzo[*a*]pyrene 4,5-oxide and *trans*-4-phenyl-3-buten-2-one distinguish transferase μ from the human basic transferases and transferase π in that the latter have considerably lower specific activities with these substrates (Warholm et al., 1981a; Guthenberg & Mannervik, 1981). However, the physiological significance of these differences is not understood at present. In rat liver, glutathione transferase C_2 (Mannervik & Jensson, 1982) is the enzyme that in catalytic properties shows the greatest similarities to human transferase μ, but important differences between the enzymes do exist.

Binding Properties. Glutathione transferases, in addition to serving as enzymes, have also been considered as intracellular binding proteins and referred to as ligandin (Litwack et al., 1971). However, it has been found that the human basic transferases α–ε have comparatively low binding affinities for bilirubin (K_d = 18–110 μM) and some additional ligands. The corresponding K_d values of the rat transferases binding these ligands most tightly are at least 10-fold lower (Jakoby et al., 1976). In contrast, human transferase μ was found to have high binding affinity for bromosulfophthalein (K_d = 1 μM) and bilirubin ($K_d \sim 10$ μM), matching the mentioned rat transferases.

Cholate and deoxycholate like bromosulfophthalein and bilirubin are good inhibitors of transferase μ (Table V). Cholate was earlier reported to be without inhibitory effect on the human basic transferases (Pattinson, 1981). This finding was confirmed in the present study and extended to transferase π. The inhibition effected by 0.2 mM sodium cholate was less than 1% for the basic transferases and <7% for transferase π. These results add to the differences between the three different groups of human glutathione transferases discussed above.

The hydrophobic *S*-*n*-hexylglutathione, a substrate and product analogue, was also found to be a potent inhibitor ($K_i \sim 1$ μM). The tight binding rationalizes the successful affinity chromatography of the enzyme (Warholm et al., 1981a). S-Substituted glutathione derivatives have earlier been shown to inhibit rat liver glutathione transferases (Askelöf et al., 1975; Jakobson et al., 1979b).

Thus, the results of the binding and kinetic experiments suggest that glutathione transferase μ, like transferase A_2 from rat liver (Mannervik et al., 1978), has two binding sites in the active site cavity. One binding site is highly specific for glutathione; the second site binds various hydrophobic molecules, including the electrophilic substrates. The noncompetitive inhibitory effects of bromosulfophthalein and bilirubin indicate that these compounds bind to a second hydrophobic binding site, distinct from the catalytic binding site. The nonlinear inhibition with bilirubin (Figure 5) could be explained by binding to both hydrophobic sites.

Three Groups of Human Glutathione Transferases. The data presented in this paper show that glutathione transferase μ in many respects differs from the basic transferases previously studied in human liver (Kamisaka et al., 1975), as well

as from the acidic transferase π (or ρ) found in placenta (Guthenberg & Mannervik, 1981) and erythrocytes (Marcus et al., 1978). It appears as though the basic and the acidic transferases are present in all individuals but that transferase μ exists only in some (Warholm et al., 1980). The basic transferases occur in separable but functionally similar forms ("α–ϵ type") (Kamisaka et al., 1975; Warholm et al., 1980). Some indications for the presence of more than one acidic ("π/ρ type") glutathione transferase have also appeared (Warholm et al., 1981b; Koskelo & Valmet, 1980), but multiple forms with a near-neutral ("μ type") isoelectric point have not yet been observed. Genetic evidence points to the existence of more than one gene for the human glutathione transferases but the gene products observed (Board, 1981) probably correspond to the three groups of enzymes. Transferase μ has not been found in human fetal liver, whereas both basic and acidic transferases exist in all samples examined (Warholm et al., 1981b). The lack of transferase μ in the fetus shows that the synthesis of this protein is induced later in life, but whether or not this occurs as a response to exposure of the individual to xenobiotics acting as inducers is unknown. It has been reported that the hepatic basic transferases are induced in humans by phenobarbital treatment (Fleischner et al., 1976), supporting the suggestion that induction may give rise to expression of the gene(s) for transferase μ. However, the data available for the material used in our studies of transferase μ do not allow any conclusions about possible induction. Nevertheless, the physiological consequences of the absence or presence of transferase μ may be far-reaching in view of the high activity of this enzyme with mutagens such as benzo[*a*]pyrene 4,5-oxide and styrene 7,8-oxide. It would appear that individuals possessing this enzyme have a better protection against certain mutagenic and carcinogenic challenges.

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Registry No. Glutathione transferase, 50812-37-8; 1-chloro-2,4-dinitrobenzene, 97-00-7; 1,2-dichloro-4-nitrobenzene, 99-54-7; ethacrynic acid, 58-54-8; *trans*-4-phenyl-3-buten-2-one, 1896-62-4; 1,2-epoxy-3-(*p*-nitrophenoxy)propane, 5255-75-4; styrene 7,8-oxide, 96-09-3; benzo[*a*]pyrene 4,5-oxide, 37574-47-3; cumene hydroperoxide, 80-15-9; Δ^5-androstene-3,17-dione, 571-36-8; *p*-nitrophenyl acetate, 830-03-5; glutathione, 70-18-8; bromosulfophthalein, 71-67-0; *S*-*n*-hexylglutathione, 24425-56-7; sodium deoxycholate, 302-95-4; sodium cholate, 361-09-1; bilirubin, 635-65-4.

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Synthesis and Properties of Radioiodinated Phospholipid Analogues That Spontaneously Undergo Vesicle–Vesicle and Vesicle–Cell Transfer†

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ABSTRACT: An efficient method for the synthesis and purification of a variety of iodinated phospholipid analogues is described. 1-Acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]amino]caproyl]phosphatidylcholine (^{125}I-PC) was prepared by alkylation of 1-acyl-2-(aminocaproyl)phosphatidylcholine with monoiodinated Bolton–Hunter reagent. ^{125}I-Labeled phosphatidic acid, phosphatidylethanolamine, and phosphatidylserine were produced from ^{125}I-PC by phospholipase D catalyzed base exchange in the presence of ethanolamine or L-serine. All of these lipid analogues transferred readily from donor vesicles into recipient membranes. When an excess of acceptor vesicles was mixed with a population of donor vesicles containing the iodinated analogues, approximately 50% of the ^{125}I-labeled lipids transferred to the acceptor vesicle population. In addition, under appropriate incubation conditions, these lipids were observed to transfer from vesicles to mammalian cells. Autoradiographic analysis of ^{125}I-labeled lipids extracted from the cells after incubation with vesicles at 2 °C for 60 min revealed that a large proportion of the ^{125}I-labeled phosphatidic acid was metabolized to ^{125}I-labeled diglyceride and ^{125}I-labeled phosphatidylcholine, whereas no metabolism of exogenously supplied ^{125}I-labeled phosphatidylethanolamine or ^{125}I-labeled phosphatidylcholine could be detected.

Several biochemical and biological studies have demonstrated that certain phospholipid molecules can exchange/transfer between populations of lipid vesicles and between vesicles and cells. Although this phenomenon has been shown to occur with unmodified phospholipids (Pagano & Huang, 1975; Papahadjopoulos et al., 1976; Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977a; Sandra & Pagano, 1979; De Cuyper et al., 1980), additional insight into the mechanism involved in this process has been obtained through the use of fluorescent lipid analogues (Monti et al., 1977; Roseman & Thompson, 1980).

The elegant studies of Pagano and co-workers using a variety of vesicle–vesicle (Pagano et al., 1981a) and vesicle–cell (Struck & Pagano, 1980; Pagano et al., 1982) systems have demonstrated that certain acyl chain labeled phospholipid analogues can readily transfer from synthetic phospholipid vesicles. This has been shown to occur with 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine (NBD-PC),[1] NBD-PE, and NBD-PA but not with NBD-ceramide or NBD-PG, suggesting some dependence of the phenomenon on the composition of the polar head group. Indeed, various polar head group labeled phospholipid analogues of phosphatidylethanolamine such as *N*-NBD-PE, *N*-Rh-PE, *N*-fluoresceinyl-PE (Struck & Pagano, 1980), and ^{125}I-phenylpropionyl-PE (Schroit, 1982) have been shown to be nontransferable.

Although the molecular arrangements responsible for the transfer phenomenon are not completely defined, the solubility of the lipid monomer in water is apparently a factor (Roseman & Thompson, 1980; Nichols & Pagano, 1981). Thus, lipid tranfer occurs with a variety of lyso- (Weltzien, 1979), short-chain (Martin & MacDonald, 1976; De Cuyper et al., 1980; Tanaka et al., 1980, 1983), and unsaturated phospholipids (Pagano & Huang, 1975; Duckwitz-Peterlein et al., 1977; Sandra & Pagano, 1979) and also probably results from the attachment of relatively bulky aromatic groups to phospholipid fatty acyl side chains, as in the instance of some of the fluorescent analogues (Monti et al., 1977; Roseman & Thompson, 1980).

If this is indeed the case, then one might assume that the attachment of other aromatic moieties to the 2-position fatty acid of phospholipids might similarly affect and ultimately

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[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; Boc-ON, 2-[[(*tert*-butoxycarbonyl)-oxy]imino]-2-phenylacetonitrile; ^{125}I-BHR, succinimido 3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionate, monoiodinated Bolton–Hunter reagent; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2; NH_2-PC, 1-acyl-2-(aminocaproyl)phosphatidylcholine; ^{125}I-PC, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]amino]caproyl]phosphatidylcholine; ^{125}I-PA, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]amino]caproyl]phosphatidic acid; ^{125}I-PE, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]amino]caproyl]phosphatidylethanolamine; ^{125}I-PS, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]amino]caproyl]phosphatidylserine; LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s).

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