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BINDING OF ETHACRYNIC ACID TO HEPATIC GLUTATHIONE S-TRANSFERASES IN VIVO IN THE RAT

Tadataka Yamada and Neil Kaplowitz

Gastroenterology and Research Services, VA Wadsworth Medical Center, and UCLA School of Medicine, Los Angeles, CA, U.S.A.

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Ethacrynic acid is a substrate for rat glutathione S-transferases in vitro (1,2). In their study defining the importance of the glutathione S-transferases in the hepatic metabolism and biliary excretion of ethacrynic acid, Wallin et al. (3) suggested that a small but consistent portion of the administered drug was bound selectively and covalently to the transferases. Such an association would represent a unique example of covalent binding of a drug to the metabolizing enzyme without prior microsomal enzyme activation. We have undertaken these studies, therefore, to characterize the binding of ethacrynic acid to the glutathione S-transferases and to delineate the selectivity of this binding.

MATERIALS AND METHODS

Ethacrynic acid and labeled ethacrynic acid (phenoxyacetic [2-^{14}C], 16 μCi/mg) were gifts of Merck, Sharpe & Dohme (Rahway, NJ). [^{14}C]Methyl-iodide (49 μCi/mmole) was obtained from the Amersham/Searle Corp. (Arlington Heights, IL). Glutathione (GSH) was obtained from the Sigma Chemical Co. (St. Louis, MO) and 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used in this study were readily available commerical products.

Preparation of tissues. Six male Sprague-Dawley rats, weighing approximately 300 g, were anesthetized with single intraperitoneal injections of pentobarbital (50 mg/kg) and placed on a heated operating board. Their abdomens were opened and their renal pedicles ligated. Their left external jugular veins were cannulated, and [^{14}C]ethacrynic acid (2 mg/kg, sp. act. 2 μCi/mg) was injected intravenously. Ninety min following drug administration, the animals were killed by exsanguination through the abdominal aorta. The livers were removed and homogenized with 2 parts (v/w) 0.1 M sodium phosphate, 0.25 M sucrose buffer (pH 7.4) using a Teflon pestle and a motor-driven tissue homogenizer. The homogenates were immediately centrifuged at 4° in a Beckman TJ-6 centrifuge (Beckman Instruments, Inc., Fullerton, CA) at 1000g for 15 min. The supernatant fractions were ultracentrifuged at 100,000g for 60 min in a Beckman L3-50 ultracentrifuge at 4° and the supernatant fractions thus obtained were pooled and dialyzed against three changes of 1000 ml of 0.01 M sodium phosphate buffer (pH 7.4) over 36 hr at 4°. Sixty ml of the dialysate were chromatographed on a Sephadex G-100 column (5 x 100 cm) at 4° using a 0.01 M sodium phosphate buffer (pH 7.4) for elution, and 10.2ml fractions were collected. Aliquots (0.1 ml) of the fractions were added to vials containing 10 ml Aquasol II (New England Nuclear, Boston, MA), and radioactivity was measured in a Beckman LS-3150T liquid scintillation spectrometer with quench correction using external standardization. CDNB-GSH conjugation activity of each fraction was measured (see below), and the fractions containing the bulk of enzyme activity (fractions 82-96) were pooled. Aliquots of the

pool were assayed for radioactivity, protein concentration (4), and ethacrynic acid covalent binding (see below). The rest was dialyzed at 4° against three changes of 2000 ml of 0.01 M Tris-Cl (pH 8.0), and batch adsorbed with 100 ml of DEAE cellulose (Whatman DE-52) swollen with the same buffer. The eluate thus obtained was further purified according to the method of Habig et al. (2), by dialysis at 4° against three changes of 2000 ml of 0.01 M potassium phosphate buffer (pH 6.7), chromatography on a CM cellulose (CM-52) column (1.5 x 40 cm), and elution with a 100 ml linear gradient of 0 to 200 mM KCl in the same buffer. The obtained fractions were assayed for GSH-CDNB, GSH-DCNB and GSH-methyl-iodide conjugating activities (see below), radioactivity, and conductivity.

Enzyme assays. The enzymatic reaction between CDNB and GSH was assayed using the method of Booth et al. (5) by measuring reaction product formation at 340 nm following incubation of 0.01 mM CDNB in 5% ethanol, 0.1 M sodium phosphate (pH 6.5) and 1 mM GSH with 10 µl of sample at 37°. The DCNB-GSH reaction was also followed at 340 nm following incubation of 0.1 mM CDNB, 10 mM GSH, 0.1 M sodium phosphate buffer (pH 8.0) and 50 µl of sample at 37° (5). The reaction between GSH and methyl-iodide (1.7 mM, 0.02 µCi/µmole) was incubated in 3 ml of 66 mM sodium phosphate, 2 mM EDTA buffer (pH 7.0) at 25° in the presence or absence of 200 µl of the test sample as the source of enzyme (6,7). Aliquots (0.5 ml) were taken at 0, 1, 3 and 5 min, transferred immediately to liquid scintillation vials containing 1.0 ml methanol, and then blown to dryness under a ventilated hood with gentle heating. Hot distilled water (1.0 ml) was added, followed by 10 ml Aquasol II, and the radioactivity of the non-volatile reaction product was measured as described. Under these conditions, the reaction was linear for 5 min without significant spontaneous interaction of substrates.

Measurement of covalent binding. Six aliquots (2 ml each) of the pool of Sephadex G-100 eluates containing enzyme were precipitated with 0.5 ml of 50% trichloroacetic acid, centrifuged at 3000 r.p.m. for 2 min, and the supernatant fractions decanted and assayed for radioactivity as described. The precipitates were suspended and washed with 0.5 ml aliquots of 50% trichloroacetic acid repeatedly until no further radioactivity could be detected in the supernatant fractions. Then the samples were treated in a similar fashion with repeated washes using 2 ml methanol and 2 ml chloroform. The remaining precipitates were dissolved in 1 ml of 1 N NaOH by heating at 37° for 15 min, and 200 µl aliquots were assayed for radioactivity. Blanks were prepared and counted for each set of samples in order to obtain appropriate background readings. Quench correction was by external standardization.

RESULTS

As depicted in Fig. 1, all of the [^{14}C]ethacrynic acid remaining bound to liver cytosol eluated in a single peak which coincided with the glutathione S-transferases peak as

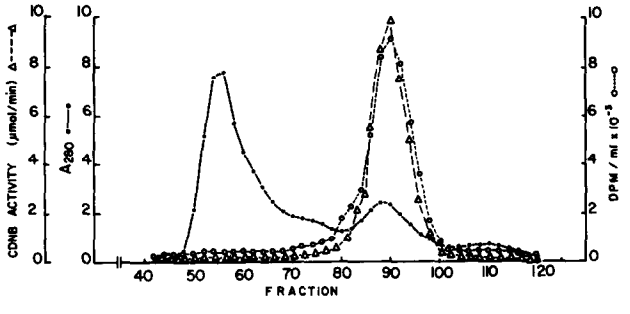

Fig. 1. Sephadex G-100 elution of liver cytosol from six rats killed 90 min following administration of [^{14}C]ethacrynic acid (2 mg/kg, sp. act. 2 µCi/mg). Sixty ml of cytosol were chromatographed on a column (5.0 x 100 cm) using 0.01 M sodium phos-

phate (pH 7.4) as elution buffer, and 10.2 ml fractions were collected. Enzyme activity, as assayed by CDNB-GSH conjugating activity (Δ), A_{280} (●), and radioactivity (o), was measured in every other fraction.

monitored by GSH-CDNB conjugating activity. The equivalent of approximately 20 percent of the administered dose of [^{14}C]ethacrynic acid was recovered in the transferase peak. As seen in Table 1, when aliquots of a pool of fractions from this peak were treated with sequential washes of trichloroacetic acid, methanol, and chloroform, approximately 13.5 percent of the radioactivity in the original enzyme pool or 2.7 percent of the administered dose of [^{14}C]ethacrynic acid remained bound. Thus, a substantial amount of ethacrynic acid appeared to covalently bind to the glutathione S-transferases.

Table 1. Covalent binding of [^{14}C]ethacrynic acid to rat liver glutathione S-transferases*

Fraction	[^{14}C]ethacrynic acid bound	
	μg/mg protein	% Initial
Pooled transferase fractions	1.76 ± 0.02	100
After trichloroacetic acid	0.56 ± 0.02	31.8 ± 1.2
After methanol	0.25 ± 0.02	14.3 ± 1.2
After chloroform (amount covalently bound)	0.24 ± 0.01	13.5 ± 0.3

*Six aliquots (2 ml each) of a pool of Sephadex G-100 eluates containing enzyme activity (Fig. 1) were precipitated with 0.5 ml of 50% trichloroacetic acid and then treated sequentially with trichloroacetic acid, methanol, and chloroform until no further radioactivity could be washed off. The precipitates were then dissolved in 1 ml of 1 N NaOH and radioactivity was measured.

With further purification, the various transferases were separated, transferases B and AA being distinct from transferases C and A by virtue of their GSH-methyl-iodide conjugating activities (Fig. 2). The fractions in Fig. 2 labeled A and C also catalyzed the DCNB-GSH reaction whereas peaks B and AA showed negligible activity (not shown). Thus, the separation of transferases closely conforms to the descriptions by Habig et al. (2). All of the radioactivity eluted with the first peak of GSH-CDNB conjugating activity, suggesting selective binding of ethacrynic acid to glutathione S-transferase C. Transferases D and E which elute before the salt gradient contained no radioactivity (not shown).

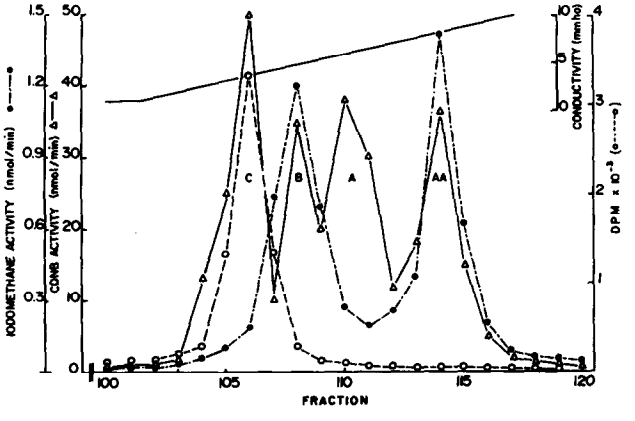

Fig. 2. Binding of [^{14}C]ethacrynic acid to glutathione S-transferase C. Fractions 82-96

from Fig. 1 were pooled, batch adsorbed with DEAE-cellulose, and then chromatographed on a CM-cellulose column (1.5 x 40 cm) and eluted with a 100 ml linear gradient of 0.01 M potassium phosphate (pH 6.7) containing 0 to 0.2 M KCl. Each fraction was assayed for conductivity (upper figure), iodomethane-GSH conjugating activity (●), CDNB-GSH conjugating activity (Δ), and radioactivity (o).

DISCUSSION

The glutathione S-transferases have three major characteristics that give rise to their potential functional importance: (1) they catalyze the enzymatic GSH-conjugation of a wide variety of substrates including many drugs and carcinogens (1,2); (2) they are capable of binding many non-substrate ligands such as bilirubin and indocyanin green (8) giving rise to the hypothesis that they function as intracellular transport carriers or storage proteins; and (3) they form covalent interactions with reactive metabolites of certain carcinogens such as 3-methylcholanthrene and azo-dyes (9,10), thus serving a potential role as important detoxification proteins. Ethacrynic acid has been shown previously to interact with the glutathione S-transferases in the first capacity, as a substrate for GSH-conjugation (1,3). Our present studies indicating selective and, in part, covalent binding describe additional interactions between ethacrynic acid and the glutathione S-transferases.

A substantial proportion (13.5%) of ethacrynic acid bound to the glutathione S-transferases was bound covalently. Since there is no evidence that ethacrynic acid is activated by microsomal enzymes prior to binding, this covalent binding is unique. The apparent specificity of this binding for transferase C is of particular interest since the majority of ethacrynic acid-GSH conjugating activity is present in transferases AA and B, although transferase C does have some of this enzyme activity (2,11). Recent studies suggest that non-substrate ligands bind to the glutathione S-transferases at sites separate from the substrate binding sites (8,12) and that substrates for one of the transferases may function as non-substrate ligands for other transferases (2,13). It is conceivable that ethacrynic acid was covalently bound to glutathione S-transferase C as a non-substrate ligand in a similar fashion. Alternatively, we cannot exclude the possibility that ethacrynic acid arlyated the enzyme at its binding site with resultant destruction and loss of enzyme activity. We estimate that at most one-third of transferase C was covalently bound, assuming only 1 molecule of ethacrynic acid bound per molecule of enzyme. Thus, the ample GSH-CDNB activity retained by our transferase C fractions could be accounted for by the enzyme remaining unbound.

Previous studies (3) have shown that, 90 min after injection of ethacrynic acid, greater than 95 percent of the drug has been cleared from the plasma and that the bulk of ethacrynic acid retained in the liver is unconjugated. We recovered 20 percent of the administered ethacrynic acid in the liver at 90 min. This suggests that ethacrynic acid binding in the liver in part reflects *in vivo* storage of the drug.

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