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Donor substrate specificity of bovine kidney gamma-glutamyltransferase

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ABSTRACT

The enzyme γ-glutamyltransferase (GGT) catalyzes the hydrolysis of the γ-glutamyl isopeptide bond of glutathione conjugates (donor substrates), releasing glutamic acid, or the transfer of the donor's γ-glutamyl group to an acceptor substrate, such as a dipeptide. The specificity of GGT for xenobiotic donor substrates has not been fully characterized. The transpeptidation activity of bovine kidney GGT was measured with glycylglycine as the acceptor substrate and several glutathione conjugate donor substrates, representative of detoxication products of polycyclic aromatic xenobiotics. HPLC separation with UV detection was used for quantitation. The commonly-used chromogenic substrate γ-glutamyl-*p*-nitroanilide was also tested. Michaelis constants (K_m) were obtained for 4-nitrobenzylglutathione (0.075 mM), 2,4-dinitrophenylglutathione (0.30 mM), 4-methylbiphenylylglutathione (0.12 mM), 1-menaphthylglutathione (0.23 mM), and 9-methylanthracenylglutathione (0.22 mM), indicating a trend towards higher values for bulkier substrates. These results provide insight into the capacity of GGT to act in the biotransformation of aromatic compounds, many of which are of toxicological importance.

1. Introduction

The goal of this investigation is to characterize the kinetics and specificity of the γ-glutamyltransferase (GGT)-catalyzed hydrolysis of aromatic glutathione (GSH) conjugates.

The presence of mercapturic acids as excretion products of aromatic compounds in mammalian urine has been known since the 19th Century [1] and was indeed recognized long before the discovery of glutathione. Mercapturic acid metabolites of many aromatic compounds, especially benzene derivatives, have been characterized in rat urine, *e.g.*, *S*-(trichlorophenyl)-*N*-acetylcysteine as a metabolite of 1,2,4-trichlorobenzene [2]; *S*-(5-chloro-2-nitrophenyl)-*N*-acetylcysteine as a metabolite of 2,4-dichloro-1-nitrobenzene [3]; and benzylmercapturic acid and *o*-, *m*- and *p*-toluylmercapturic acids as metabolites of toluene [4].

Xenobiotic-derived mercapturic acids are found in human urine and have been applied as biomarkers of occupational exposure. For example, *N*-acetyl-*S*-(*n*-propyl)-L-cysteine (*n*-propyl mercapturic acid) was found in the urine of workers exposed to 1-bromopropane, used as an industrial degreasing agent [5]. *S*-*p*-Toluylmercapturic acid was detected in the urine of workers exposed to the aromatic solvent toluene [6]. Recent advances in HPLC-mass spectrometry are opening a new window, with regard to human urine analysis. For example, Sabatini and colleagues applied triple-quadrupole LC-MS-MS to detect phenyl-mercapturic acid, benzylmercapturic acid, and *o*-methylbenzylmercapturic acid at μg per g creatinine levels in urine samples of traffic wardens, presumably due to exposure to ambient levels of benzene, toluene, and xylenes [7]; Reska et al. have developed an LC-MS-MS method for quantitation of the styrene-derived mercapturic acids *S*-(2-hydroxy-1-phenylethyl)- and acids *S*-(2-hydroxy-2-phenylethyl)-*N*-acetylcysteine, and found the levels to be higher in cigarette smokers [8]; Egner et al. applied HPLC isotope-dilution triple-quadrupole mass spectrometry to quantitate sulforaphane mercapturic acid derived from dietary glucosinolates [9].

Despite the toxicological importance of polycyclic aromatic compounds, the details of their GSH-dependent metabolism to

Abbreviations: BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; Cys, cysteinyl; Cys-Gly, cysteinylglycine; DNP, 2,4-dinitrophenyl; DNP-GSH, 2,4-dinitrophenylglutathione; GGT, γ-glutamyltransferase, γ-glutamyltranspeptidase; Gly-Gly, glycylglycine; GpNA, γ-glutamyl-*p*-nitroanilide; GSH, glutathione; HPLC, high performance liquid chromatography; M, 1-menaphthyl; MCl, 1-chloromethylnaphthalene, 1-menaphthylchloride; M-GSH, 1-menaphthylglutathione; MA, 9-methylanthracene; MACl, 9-chloromethylanthracene; MA-GSH, 9-methylanthracenylglutathione; MBP, 4-methylbiphenyl; MBPBr, 4-bromomethylbiphenyl; MBP-GSH, 4-methylbiphenylyglutathione; NB, 4-nitrobenzyl; 2-NBA, 2-nitrobenzoic acid; NBCl, 4-nitrobenzylchloride; NB-GSH, 4-nitrobenzylglutathione; TCA, trichloroacetic acid; X-Cys, cysteine conjugate; X-Cys-Gly, cysteinylglycine conjugate; X-GSH, glutathione conjugate.

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mercapturic acids are not well understood. *S*-(1-Hydroxy-1,2-dihydro-2-naphthalenyl)-*N*-acetylcysteine was identified as a major urinary metabolite of naphthalene in mice [10]. Yang and colleagues collected urine from rats administered intraperitoneal benzo[*a*]pyrene (BP) and identified, by collision-induced dissociation mass spectrometry, the mercapturic acid 7,8,9,10-tetrahydro-8,9,10-trihydroxybenzo[*a*]pyrene-7-*S*-(*N*-acetyl)cysteine, presumably derived from the reaction of GSH with BP-7,8,9,10-tetrahydro-9,10-dihydroxy-7,8-epoxide [11]. This "reverse" BP dihydrodiol epoxide (BPDE) is considered less biologically active (mutagenic/ carcinogenic) than the bay-region isomer BP-7,8,9,10-tetrahydro-7,8-dihydroxy-9,10-epoxide. Hecht and colleagues examined phenanthrene-derived mercapturic acids in human smokers' urine, and again found only a mercapturic acid metabolite of the "reverse" dihydrodiol epoxide, phenanthrene 1,2,3,4-tetrahydro-3,4-dihydroxy-1,2-epoxide, and not the bay-region 1,2,3,4-tetrahydro-1,2-dihydroxy-3,4-epoxide [12]. They noted that "there does not appear to be a single report in the literature that has investigated the presence in human urine of a PAH diol epoxide-derived mercapturic acid", and concluded that their findings "call into question the widely held assumption, based completely on *in vitro* studies, that bay region diol epoxides of carcinogenic PAH are detoxified by GSTs. This assumption underlies multiple literature studies that have investigated polymorphisms in GST enzymes ... with respect to cancer outcomes in people exposed to PAH, such as smokers. Our results demonstrate that this assumption is untenable." A subsequent study using human hepatocytes supported the interpretation that GSH conjugation is a significant route for detoxication of the less toxic "reverse" dihydrodiol epoxide but not the critical bay region dihydrodiol epoxides [13].

The metabolism of a xenobiotic to a mercapturic acid requires a series of enzymatic steps, beginning with bioactivation to an electrophilic species (such as a quinone or an epoxide) and glutathione transferase-catalyzed conjugation with GSH, followed by: (i) hydrolysis of the γ-glutamyl isopeptide bond of the GSH conjugate, catalyzed by γ-glutamyltransferase (GGT; γ-glutamyltranspeptidase; EC 2.3.2.2); (ii) hydrolysis of the peptide bond of the resulting cysteinylglycine (Cys-Gly) conjugate, catalyzed by a dipeptidase; (iii) N-acetylation of the cysteine (Cys) conjugate to give the mercapturic acid. The literature on *in vitro* processing of aromatic GSH conjugates to mercapturic acids is surprisingly sparse. A recent study described the hydrolysis of several aryl cysteinylglycine conjugates catalyzed by porcine kidney membrane dipeptidase [14]. Research on the final step in the mercapturic acid pathway will be facilitated by the recent identification of the major mammalian cysteinyl-S-conjugate N-acetyltransferase enzyme, N-acetyltransferase 8 [15]. The goal of the current study is a systematic examination of the action of GGT on aromatic GSH conjugates.

GGT is a membrane-bound heterodimeric glycoprotein present in organisms ranging from bacteria to humans [16]. In mammals, the enzyme is found mainly in the kidney [17,18]. The heavy subunit of GGT anchors the protein to the cell membrane and the light subunit contains the catalytic site of the enzyme [19]. GGT acts in the metabolism of xenobiotics and endogenous GSH conjugates, in GSH homeostasis and in cysteine recovery, and it is often used as a biomarker of liver damage [18–22].

The catalytic cycle of GGT follows a ping-pong mechanism [23,24]. In the first step, the γ-glutamyl moiety of the donor substrate (*e.g.*, GSH or GSH conjugate) reacts with a hydroxyl group in the active site of the enzyme to generate a tetrahedral acyl-enzyme intermediate [22,23]. In the case of *Escherichia coli* GGT, the essential catalytic residue has been identified as threonine-391 [25,26]. Release of the donor in its free amine form (*e.g.*, Cys-Gly) from the tetrahedral intermediate produces the covalent acyl-enzyme (γ-glutamyl enzyme) intermediate [22,23]. Subsequent acyl transfer of the γ-glutamyl moiety to an acceptor substrate, such as water or a dipeptide, releases glutamate or a γ-glutamyl dipeptide, respectively, and completes the reaction cycle [22–24,27,28]. Both the hydrolysis (acceptor substrate, water) and transpeptidation (acceptor substrate, dipeptide) modes of GGT catalysis are believed to have physiological importance [29].

Despite many studies on the physiology of GGT, including its use as a serum marker of hepatotoxicity and xenobiotic exposure [30], there is still much to learn about the role of this enzyme in the mercapturic acid pathway. Analysis of the human genome has recently revealed the existence of a family of GGT genes with as many as 13 members [21], and it is not yet clear how many of these genes are expressed as functional enzymes.

Most biochemical analyses of GGT employ colorimetric assays with GPNA or other chromogenic substrates [31–34]. Cook and colleagues measured the donor substrate specificity of rat kidney GGT [35]. The researchers tested several γ-glutamyl compounds, and noted that the K_m and V_{max} values varied greatly. They speculated that differences in K_m were primarily due to effects on the rate constant k_3 for conversion of the initial donor substrate-enzyme complex to the γ-glutamyl-enzyme intermediate [35]. (The Michaelis constant is a measure of both enzyme-substrate binding affinity and of the kinetics of the subsequent product formation steps [36].) Differences in V_{max} were thought to be due to the electron-withdrawing ability of the adduct; faster hydrolysis occurred with more electron-withdrawing adducts, "due to destabilization of the γ-peptide bond, making formation of the γ-glutamyl enzyme intermediate easier". It was concluded that the γ-glutamyl moiety of the donor substrate is the primary determinant recognized by GGT; however, the chemical nature of the adduct was important in determining the rate of the formation of the γ-glutamyl-enzyme intermediate [35]. Srivastava et al. [37] showed that the GSH conjugate of (+)-*anti*-BPDE is a substrate for a commercially-available GGT preparation, but detailed kinetic studies were not reported. Wickham et al. [22] expressed human GGT1 and GGT5 in *Pichia pastoris* and carried out kinetic studies with several substrates, including GSH, GSSG, and NB-GSH. The hydrolysis mode of GGT was assayed, using a coupled chromogenic assay for glutamate release.

Notwithstanding the work cited above, no systematic study of the effects of aromatic S-substituents on GGT activity has been reported. To elucidate further the substrate specificity of GGT, we have tested bovine kidney GGT with several GSH conjugates representative of detoxication products of aromatic xenobiotics. Enzyme kinetic studies were performed to investigate structural factors affecting GGT activity.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA; 98%), 1-(chloromethyl)naphthalene (MCl; menaphthyl chloride;>97%), L-glutamic acid-γ-(4-nitroanilide) (GpNA; γ-glutamyl-*p*-nitroanilide), GSH (>98%), 2-nitrobenzoic acid (2-NBA; 95%), 4-nitrobenzyl chloride (NBCl; 99%), and trichloroacetic acid (TCA) solution (6.1 N) were obtained from Sigma Aldrich (St. Louis, MO, USA). 4-(Bromomethyl)biphenyl (MBPBr; 96%) and glycylglycine (Gly-Gly; 99%) were purchased from Acros Organics (Bridgewater, NJ, USA). 1-Chloro-2,4-dinitrobenzene (CDNB; 98%) and 9-(chloromethyl)anthracene (MACl; 98%) were obtained from Alfa Aesar (Ward Hill, MD, USA). Bovine kidney GGT was purchased from Lee Biosolutions, Inc. (Brentwood, MO, USA). All other chemicals and reagents were the highest grades commercially available.

GSH conjugates were prepared and purified as described previously [14]. The conjugates were thoroughly washed with water