Phospholipid hydroperoxide glutathione peroxidase activity of human glutathione transferases

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Human glutathione transferases (GSTs) from Alpha (A), Mu (M) and Theta (T) classes exhibited glutathione peroxidase activity towards phospholipid hydroperoxide. The specific activities are in the order: GST A1-1 > GST T1-1 > GST M1-1 > GST A2-2 > GST A4-4. Using a specific and sensitive HPLC method, specific activities towards the phospholipid hydroperoxide, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OOH) were determined to be in the range of 0.8–20 nmol/min per mg of protein. Two human class Pi (P) enzymes (GST P1-1 with Ile or Val at position 105) displayed no activity towards the phospholipid hydroperoxide. Michaelis–Menten kinetics were followed only for glutathione, whereas there was a linear dependence of rate with PLPC-OOH concentration. Unlike the selenium-

dependent phospholipid hydroperoxide glutathione peroxidase (Se-PHGPx), the presence of detergent inhibited the activity of GST A1-1 on PLPC-OOH. Also, in contrast with Se-PHGPx, only glutathione could act as the reducing agent for GST A1-1. A GST A1-1 mutant (Arg15Lys), which retains the positive charge between the GSH- and hydrophobic binding sites, exhibited a decreased k_{cat} for PLPC-OOH but not for CDNB, suggesting that the correct topography of the GSH site is more critical for the phospholipid substrate. A Met208Ala mutation, which gives a modified hydrophobic site, decreased the k_{cat} for CDNB and PLPC-OOH by comparable amounts. These results indicate that Alpha, Mu and Theta class human GSTs provide protection against accumulation of cellular phospholipid hydroperoxides.

INTRODUCTION

The glutathione transferases (GSTs; EC. 2.5.1.18) are a complex family of enzymes involved in detoxification of a wide range of harmful chemicals, including environmental pollutants, carcinogens, mutagens and toxic products such as lipid hydroperoxides generated during oxidative stress [1–3]. The lipid peroxidation products formed by the free-radical-mediated attack on membrane lipids can propagate an autocatalytic chain of lipid peroxidation in the presence of oxygen, eventually leading to membrane destruction [4,5]. Lipid peroxidation products can also cause DNA damage [3]. Hence, the prevention of lipid peroxidation is an essential process in all aerobic organisms. Previously many studies investigating lipid peroxidation have examined GST activity towards substrates such as *t*-butyl or cumene hydroperoxides. Some physiologically relevant substrates such as phospholipid hydroperoxides have also been used to measure the activity of certain GSTs, such as the rat Alpha (A) class [6], rat liver microsomal [7–9], mouse lung [10], bovine cornea and retina [11], human liver microsomal [12] and human Alpha class GSTs [13–16]. There are no reports on the activity of human GST Mu (M), Pi (P) or Theta (T) on phospholipid hydroperoxides, and no reports have compared the GSTs under identical conditions using a reliable assay, or reported on the mechanism of reduction. We have previously reported an assay that is based on separation and quantification of phospholipid hydroperoxides and the corresponding alcohol products. This method is more sensitive and subject to much less interference from contaminating substances than the coupled spectrophotometric assay [17]. This present paper describes its application to

determining the activity of some human cytosolic GST isoenzymes towards a phospholipid hydroperoxide and the mechanism of action of the most active GST.

EXPERIMENTAL

Materials

1-Palmitoyl-2-linoleoyl-L-3-phosphatidylcholine (PLPC), soya-bean lipoxidase (EC.1.13.11.12; type IV), choline chloride, 1-chloro-2,4-dinitrobenzene (CDNB), deoxycholic acid and GSH were purchased from Sigma (Poole, Dorset, U.K.). Methanol and acetonitrile, of HPLC grade, were filtered and degassed before use. All other reagents were of analytical grade and available commercially.

Enzyme purification

All GSTs used were recombinant enzymes expressed heterologously in *Escherichia coli*. The expression and purification of GSTs A1-1, A4-4, P1-1 (Ile-105), M1-1 (allelic variant b) and T1-1 have been described previously [18–22]. Expression and purification of GST A2-2 (K. Svensson, M. Widersten and B. Mannervik, unpublished work) and P1-1 (Val-105) (A.-S. Johansson, G. Stenberg, M. Widersten and B. Mannervik, unpublished work) were performed essentially as for GST A1-1 [18]. Concentrations of purified enzymes were determined spectrophotometrically at 280 nm, using absorption coefficients obtained by amino acid analysis of the different isoenzymes.

Abbreviations used: GST, glutathione transferase; A, Alpha; T, Theta; M, Mu; P, Pi; CDNB, 1-chloro-2,4-dinitrobenzene; PLPC, 1-palmitoyl-2-linoleoyl-L-3-phosphatidylcholine; PLPC-OOH, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine; PLPC-OH, 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine; Se-PHGPx, selenium-dependent phospholipid hydroperoxide glutathione peroxidase; G-site, GSH-binding site; H-site, hydrophobic binding site.

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Preparation and purification of 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OOH)

PLPC-OOH was prepared from PLPC using soya-bean lipoxidase as described by Maiorino et al. [24]. The PLPC-OOH was separated from unoxidized phospholipid by FPLC on a PepRPC HR10/10 column with a gradient of water (100%) to methanol (100%) in 20 min. The concentration of the phospholipid hydroperoxide collected in 100% methanol was determined by absorbance at 232 nm ($\epsilon = 25000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [25]. PLPC-OH standards were prepared as described by Bao et al. [17].

Enzyme assay

GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) was determined by the method of Habig et al. [26]. The assay mixture contained (in 1 ml) 1 mM CDNB, 1 mM GSH and 0.1 M potassium phosphate, pH 6.5; the rate of increase in absorbance at 340 nm was monitored at 25 °C for 3–5 min after the addition of the appropriate amount of enzyme. During this period, the rate of reaction was linear with time. To investigate competition between PLPC-OOH and CDNB, PLPC-OOH (100 μM) was added to the assay mixture containing an equal concentration of CDNB.

HPLC assay

GST activity towards PLPC-OOH was determined using a sensitive and specific HPLC method as described by Bao et al. [17]. The assay mixture contained 0.1 M Tris/HCl, pH 7.4, 2 mM EDTA, 1 mM NaN_3 , 3 mM GSH, 25 μM PLPC-OOH and the appropriate amount of enzyme in a final volume of 0.5 ml. The reaction was carried out at 37 °C, and termination was achieved by the addition of ice-cold acetonitrile. Before HPLC analysis, the samples were centrifuged at 11600 g for 2 min. An Ultracarb 5 ODS (20) column (250 mm $\times$ 4.6 mm) at 30 °C was used to separate PLPC-OOH and 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OH). The mobile phase consisted of acetonitrile, methanol and water (50:49:0.5, by vol.) containing 10 mM choline chloride. The flow rate was maintained at 0.5 ml/min, and detection was at 232 nm. Conversion of substrate into product was determined from the peak height of the PLPC-OH standards (see above). The linear range of PLPC-OH formation was up to 25%.

Kinetic studies

The K_m and k_{cat} values of the GSTs for GSH with CDNB or PLPC-OOH as electrophilic substrates were determined by triplicate measurements of activity at various concentrations (0.1–4 mM) of GSH. The CDNB and PLPC-OOH were added at fixed concentrations of 1 mM and 25 μM respectively. Data analysis of the results was carried out using the method of Wilkinson [27].

RESULTS AND DISCUSSION

Reaction of GSTs with PLPC-OOH

Figure 1 displays a chromatogram of PLPC-OOH and PLPC-OH used to determine phospholipid hydroperoxide glutathione peroxidase activity. Under the specific HPLC conditions employed (see the Experimental section), the retention times of PLPC-OOH and PLPC-OH were 18.3 and 19.8 min respectively. The glutathione peroxidase activity of various GSTs towards

Figure 1 Separation of PLPC-OOH and PLPC-OH by HPLC

PLPC-OOH (25 μM) was incubated in 0.1 M Tris/HCl buffer, pH 7.4, with GSH (3 mM) alone (**A**), or with GSH (3 mM) and human GST T1-1 (10 μg) (**B**), at 37 °C for 15 min. The elution positions of the standards are indicated by arrows. The small peak between PLPC-OOH and PLPC-OH is probably a positional isomer of PLPC-OOH.

Table 1 Specific activities of various human GSTs towards phospholipid hydroperoxide and CDNB

Specific activities are the means of at least triplicate results $\pm$ S.D. PLPC-OOH activity was determined at 25 μM PLPC-OOH/3 mM GSH, 37 °C, pH 7.4. CDNB activity was determined at 1.0 mM CDNB/1.0 mM GSH, 25 °C, pH 6.5. These conditions give different values for some isoenzymes compared with those previously published [3]. n.d., not detectable (activity less than 0.02 nmol/min per mg of protein).

Human GST	Specific activity (nmol/min mg of protein)		Ratio of activities (PLPC-OOH:CDNB)
	PLPC-OOH	CDNB	
A1-1	18.7 ± 2.1	23400 ± 2100	0.8×10^{-3}
A2-2	2.1 ± 0.3	14200 ± 1700	0.15×10^{-3}
A4-4	0.8 ± 0.1	3600 ± 200	0.21×10^{-3}
M1-1	2.3 ± 0.3	127000 ± 4000	0.18×10^{-6}
P1-1 (Ile-105)	n.d.	52400 ± 800	0
P1-1 (Val-105)	n.d.	30800 ± 900	0
T1-1	3.5 ± 0.4	< 260	> 0.014

PLPC-OOH, together with the activities towards CDNB, are shown in Table 1. All values were corrected for the blank rate with no GST enzyme added. Human GST A1-1 had the greatest activity towards PLPC-OOH, followed by GST T1-1, M1-1, A2-2 and finally A4-4. The two variants of GST P1-1 displayed no activity towards PLPC-OOH but had relatively large activities

Table 2 Kinetic parameters for GSH with CDNB or PLPC-OOH as a substrate for GST A1-1, M208A and R15K

Values for K_m and k_{cat} are the means of triplicate measurements for GST activity $\pm$ S.D. at various concentrations of GSH. PLPC-OOH and CDNB were added to the reactions at fixed concentrations of 0.025 and 1 mM respectively. Values for $k_{cat}/K_m^{PLPC-OOH}$ were calculated from the rate of reaction of GST A1-1 with 3 mM GSH and a range of [PLPC-OOH] from 0.01–0.3 mM. The conditions for the CDNB and PLPC-OOH assays are described in the Experimental section. Values for k_{cat}/K_m^{GSH} are probably underestimated due to the non-saturating level of the second substrate.

Substrate	GST A1-1 enzyme	K_m^{GSH} (mM)	k_{cat} (s^{-1})	apparent k_{cat}/K_m^{GSH} ($mM^{-1} \cdot s^{-1}$)	$k_{cat}/K_m^{PLPC-OOH}$ ($\mu M^{-1} \cdot s^{-1}$)
PLPC-OOH	Wild-type	0.09 ± 0.02	7.8×10^{-3}	0.0870	4.2
	M208A	0.32 ± 0.09	3.0×10^{-3}	0.0094	1.3
	R15K	0.46 ± 0.10	4.1×10^{-3}	0.0089	2.4
CDNB	Wild-type	0.24 ± 0.05	9.8	41.0	
	M208A	2.10 ± 0.40	4.9	2.3	
	R15K	1.10 ± 0.10	9.4	8.6	

towards CDNB. Activities towards PLPC-OOH were much lower than those towards CDNB, with the exception of human GST T1-1, where the ratio was more comparable. The range of activities towards PLPC-OOH determined in this paper was from 0.8 to 20 nmol/min per mg of protein. Other studies have measured human GST A4-4 activity towards dilinoleoyl phosphatidylcholine hydroperoxide and dilinoleoyl phosphatidylethanolamine hydroperoxide, and reported greater specific activities in the range of 0.2–1.2 μ mol/min per mg of protein [13–15]. However, the latter activities were measured using different phospholipids and employing a spectrophotometric assay that is subject to interference, and only allows GSH to be tested as a substrate, whereas we used a more specific and sensitive HPLC method that directly measures product formation and consequently also allows a variety of substrates to be tested. There is one report of human liver microsomal GST activity towards phospholipid hydroperoxides [12] with values within the range of the activities described in the present report; also, the activity of rat liver microsomal glutathione transferase measured using a modification of our HPLC method [8] yielded similar GST activity values.

Mechanism of action of GST A1-1 on PLPC-OOH

The reaction of the most active enzyme, GST A1-1, was examined further. Addition of the detergent Triton X-100 [0.1% (v/v)] totally inhibited the activity of GST A1-1 on PLPC-OOH. In contrast, addition of Triton X-100 increases the activity of Se-PHGPx by more than eightfold [16]. Kinetic parameters for glutathione with either CDNB or PLPC-OOH as substrates were determined (Table 2). The kinetics of GST A1-1 on PLPC-OOH showed that reduction of PLPC-OOH did not follow Michaelis–Menten kinetics, and a range of PLPC-OOH concentrations from 10 to 300 μ M against enzyme rate gave a linear dependence. This is consistent with some other lipid hydroperoxides, such as 1-linoleoyl-2-palmitoyl phosphatidylcholine, 2-linoleoyl-1-palmitoyl phosphatidylethanolamine and dilinoleoyl phosphatidylcholine [8]. The K_m and k_{cat} values for GSH with CDNB are not directly comparable with values with PLPC-OOH as the second substrate, since PLPC-OOH could not be saturating.

We also determined whether other thiols (1 mM) could substitute for GSH. GST A1-1 activity on PLPC-OOH was not observed when cysteine or cysteinyl glycine was substituted for

GSH in the reaction, and very low activities of GST A1-1 (< 5% of the activity with GSH) were observed when homocysteine or dithiothreitol was substituted for GSH at equivalent concentrations. Hence, GSH is the preferential substrate for GST A1-1, whereas, for Se-PHGPx, a variety of different substrates may be substituted for GSH, and the greatest activity on PLPC-OOH was observed with dithiothreitol [17].

Possible competition between CDNB and PLPC-OOH for the binding sites on GST A1-1 was tested. PLPC-OOH was not an inhibitor of CDNB (100 μ M) conjugation by GSTA1-1, up to a concentration of 100 μ M PLPC-OOH. However, only CDNB follows saturation kinetics. This may indicate that CDNB and PLPC-OOH binding are not mutually exclusive, but may allow the simultaneous presence of the two substrates at the active site. The results may also indicate that the binding of PLPC-OOH is too weak to compete with CDNB.

The interaction was further examined using GST A1-1 mutants, Arg15Lys (R15K) and Met208Ala (M208A). The mutation R15K affects both the GSH-binding site (G-site) and the hydrophobic binding site (H-site) [28], and the M208A modification affects the H-site [29]. The R15K mutant retains the positive charge between the G- and H- sites, and the mutant exhibited a decreased k_{cat} for PLPC-OOH but not for CDNB, suggesting that the correct topography of the GSH site is more critical for the phospholipid substrate. Both mutants displayed a decrease in specific activity towards PLPC-OOH and CDNB, but the magnitude of the change was different for each substrate; the activities towards PLPC-OOH and CDNB were decreased to 50–60% and 20–30% of the wild-type activity respectively (results not shown). The apparent K_m values for GSH increased for both of the mutants, and k_{cat} and k_{cat}/K_m^{GSH} decreased, except for the R15K mutant with CDNB as the substrate, where no significant decrease in catalytic activity was observed (Table 2). The effect of the mutations on GST A1-1 activity towards PLPC-OOH may be partly ascribed to the approx. fourfold increase in K_m^{GSH} (Table 2). However, the mutations do not affect the $k_{cat}/K_m^{PLPC-OOH}$ to a large degree.

Contribution of GSTs to cellular activity on phospholipid hydroperoxides

Overall, the activity described in the present report of the Alpha, Mu and Theta GSTs towards phospholipid hydroperoxide indicates that they may play an important role in the protection against the toxic products generated during lipid peroxidation. The apparently lower activity of the various GST isoenzymes with the phospholipid hydroperoxide in comparison with Se-PHGPx may be balanced by the fact that GST enzymes are usually more abundant in human tissues. In human liver, GSTs may constitute as much as 5% of the total soluble protein [2]. The most active Alpha class GSTs constitute a major portion of GST protein of human liver and kidney, and in individuals having a null phenotype for the polymorphic GST M1-1 (50% of the population [30]), the Alpha class GSTs constitute a major fraction of protein and activity of liver GSTs [31]. Thus individuals with higher GST levels may have better protection against lipid peroxidation. In addition to this, when dietary selenium is low, the activity of other antioxidant enzymes, such as GSH peroxidase and Se-PHGPx, may decrease [32–35] and hence the reduction of phospholipid hydroperoxides by GSTs becomes more important.

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