Glutathione S-transferases Y_cY_{fetus} and Y_cY_c — kinetic and inhibitor studies relating to their glutathione peroxidase activities

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Summary

The kinetic properties of the glutathione (GSH) peroxidase activities of GSH S-transferases Y_cY_{fetus} and Y_cY_c were compared. The catalytic efficiency of the fetal iso-enzyme with cumene hydroperoxide as substrate was approximately four times higher than the other. The effects of the non-substrate ligand rose-Bengal as well as the substrate ligands sulphobromophthalein and acrolein on the GSH peroxidase activity of these two iso-enzymes were also investigated. Depending on the ligand, the inhibition profiles of these two iso-enzymes when measured with either the peroxidase substrate, cumene hydroperoxide or the standard GSH S-transferase substrate 1-chloro-2,4-dinitrobenzene were found to be either very similar (sulphobromophthalein) or markedly different (rose Bengal and acrolein). Significantly, the GSH peroxidase activity of the fetal iso-enzyme was far less susceptible to inhibition by the teratogen, acrolein, than that of the Y_cY_c iso-enzyme. It is therefore attractive to suggest that should a similar situation arise *in vivo*, this resistance to peroxidase inhibition may play a role in preventing the fetotoxic effects of acrolein.

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The glutathione (GSH) S-transferases (EC 2.5.1.18) are a family of multifunctional enzymes which play an important role in the metabolism of a wide variety of xenobiotics by catalysing the conjugation of GSH with the electrophilic centres of these xenobiotics.[1] The GSH S-transferases also exhibit selenium-independent GSH peroxidase activity for organic hydroperoxides[2] and they may have an important biological function in the protection of membranes from lipid peroxidation.

The GSH S-transferases have been shown to bind, non-covalently, to numerous lipophilic substrate and non-substrate ligands. The binding of non-substrate ligands by the GSH S-transferases has suggested a potentially important role for these enzymes as storage and/or transport proteins. The binding of substrate and non-substrate ligands also serves to inhibit the enzyme activity of the GSH S-transferases towards the standard electrophilic substrates,[3,5-9] which may have important toxicological implications. Most inhibitor studies to date have been performed using 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate and few data are available on the effects of substrate and non-substrate ligands on the GSH peroxidase activity of the GSH S-transferases.

We recently described[10] the identification of a novel fetal rat liver GSH S-transferase iso-enzyme with high GSH peroxidase activity, namely GSH S-transferase Y_cY_{fetus}. In the study reported here we compared the kinetic properties of the GSH peroxidase activity of the two GSH S-transferase iso-enzymes, Y_cY_{fetus} and Y_cY_c. The effects of the non-substrate ligand, rose Bengal, as well as the substrates sulphobromophthalein (BSP) and acrolein (the latter recently identified by us as a non-competitive inhibitor of the GSH S-transferases[9]), on the GSH peroxidase activity of these two iso-enzymes were also investigated. For comparative purposes, the effects of these ligands on GSH S-transferase activity using CDNB as the electrophilic substrate were also assessed. The Y_cY_c iso-enzyme was chosen for comparison with the fetal iso-enzyme, since the Y_c sub-unit is associated with the high GSH peroxidase activity of the major basic adult rat liver GSH S-transferases.[11]

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Materials and methods

All chemicals used were of analytical grade. GSH S-transferases Y_cY_{fetus} and Y_cY_c were purified as previously described.[10]

Enzyme assays

GSH peroxidase activity was measured using the coupled assay of Prohaska and Ganther,[2] as previously described.[10] The reaction mixture, in a cuvette, consisted of 0,54 ml 0,1M potassium phosphate buffer (pH 7,0)/0,1M KCl; 0,1 ml 0,03M ethylenediamine tetra-acetic acid (EDTA); 0,1 ml 0,01M GSH; 0,1 ml 0,0011M NADPH and 0,1 ml GSH reductase (4 μg of protein, i.e. an excess). The enzyme (0,05 ml) was added to the above mixture, which was then allowed to incubate for 10 minutes at 25°C before initiation of the reaction by the addition of 0,01 ml peroxide solution. A blank contained all of the above with the enzyme replaced by its solute buffer. After a delay time of 0,3 minute, the change in absorbance at 340 nm was recorded for 1 minute on a double-beam spectrophotometer and the enzyme activity was automatically calculated from the slope of the line as μmol NADPH oxidised/min/ml. All solutions and buffers were prepared using distilled, de-ionised water. Solutions, except the phosphate buffer and EDTA, were freshly prepared each day. The enzyme was diluted with 0,01M potassium phosphate buffer (pH 7,0) so that the amount added to the assay system provided an absorbance change of $\leqslant$ 0,05/min (0,83 μg Y_cY_{fetus} iso-enzyme and 1,58 μg Y_cY_c iso-enzyme). Varying concentrations of cumene hydroperoxide (80% in cumene) were prepared in 95% (v/v) ethanol. This resulted in a total ethanol concentration in the assay of less than 1%.

Kinetic and inhibitor studies

The initial rates of the GSH peroxidase reaction were measured at a cumene hydroperoxide concentration of 0,5 mM for varying concentrations of GSH, and at a GSH concentration of 1,0 mM for varying concentrations of cumene hydroperoxide. The kinetic data were analysed by a non-linear regression computer program. In the GSH peroxidase inhibitor

studies, 10 μl of an appropriate amount of the inhibitor was added to the reaction mixture just before the 10-minute incubation period. These additions did not affect the pH of the reaction mixture significantly. Oxidised GSH (GSSG) (0,1 mM) was added after the assays to cuvettes in which inhibitors were used to ensure that GSH reductase had not been inhibited, and consequently limiting to the overall reaction. The effects of ionic strength on inhibition were determined by increasing the KCl concentration in the potassium phosphate buffer used in the reaction mixture. The KCl concentrations in the buffer were 0, 0,1, 0,2, 0,3 and 0,5M, which corresponded to total added KCl concentrations in the reaction mixture of 0, 54, 108, 162 and 270 mM, respectively. The GSH S-transferase inhibitor studies using CDNB as enzyme substrate were performed as previously described for acrolein.[9] Acrolein and rose Bengal solutions were prepared in ethanol (95%), and BSP solutions were prepared in H_2O. For the kinetic and inhibitor studies the assays were performed in triplicate. The concentration of inhibitor resulting in 50% inhibition of enzyme activity (I_{50} value) was determined from plots of remaining activity v. inhibitor concentration. Control values were obtained by pre-incubation with an appropriate volume of solvent in the absence of inhibitor.

Results

The velocity v. substrate concentration curves for both iso-enzymes gave the hyperbola typical for reactions obeying Michaelis-Menten kinetics, and double reciprocal or Lineweaver-Burk plots for the reactions showed the expected linearity (data not shown). The apparent K_m values for GSH for transferases Y_cY_{fetus} and Y_cY_c were 0,134 mM and 0,105 mM, respectively. The apparent K_m values for cumene hydroperoxide were 0,169 mM and 0,842 mM for transferases Y_cY_{fetus} and Y_cY_c, respectively.

The catalytic efficiency (expressed as K_{cat}/K_m)[12] of the two iso-enzymes for cumene hydroperoxide was determined with 0,1 mM cumene hydroperoxide, at which concentration first-order kinetics applied. Under the assumptions of Michaelis-Menten kinetics, the initial rate divided by the product of the total enzyme and substrate concentrations equals K_{cat}/K_m.[12] This ratio was determined at a GSH concentration of 1 mM, i.e. at saturating concentrations of GSH. The catalytic efficiency of GSH S-transferases Y_cY_{fetus} and Y_cY_c for cumene hydroperoxide were 64/mM/s and 16/mM/s, respectively.

The effect of pH on the enzymatic reaction showed that the non-enzymatic reaction was strongly pH-dependent (data not shown). At pH 6,0 the GSH peroxidase activity was low and increased dramatically at a pH of 7,0. At pH 8,0 the non-enzymatic rate was found to be very high and consequently a pH value of 7,0 was considered to provide an even balance between the spontaneous and enzyme-catalysed reaction rates.

Ionic strength was found to influence the enzyme-catalysed reduction of cumene hydroperoxide (data not shown). Maximum activities for the two iso-enzymes were obtained at KCl concentrations of 54 - 108 mM.

Fig. 1 shows acrolein inhibition of the GSH peroxidase and GSH S-transferase activity of the two iso-enzymes. The I_{50} values for inhibition of the GSH peroxidase activity of transferases Y_cY_{fetus} and Y_cY_c were 650 μM and 330 μM, respectively (Table I). The I_{50} values for the inhibition of the transferase activity of Y_cY_{fetus} and Y_cY_c were 700 μM and 800 μM, respectively (Table I).

The I_{50} values for inhibition of the GSH peroxidase activity of the two iso-enzymes by BSP were 50 μM and 116 μM, respectively (Table I). The I_{50} values for inhibition of the GSH S-transfererase activity of Y_cY_{fetus} and Y_cY_c by BSP were 48 μM and 114 μM, respectively (Table I), being almost identical to the values obtained for BSP inhibition of GSH peroxidase activity.

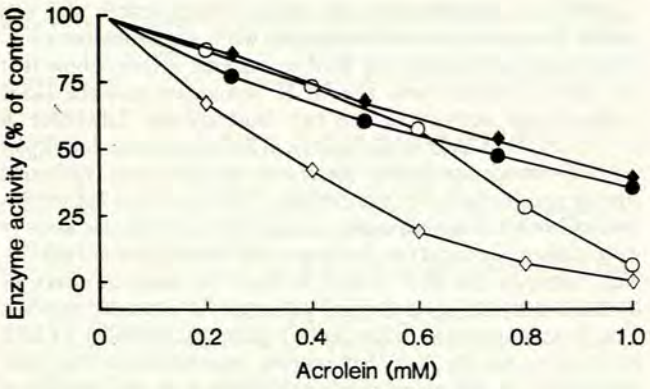

Fig. 1 Inhibition of enzyme activity by varying concentrations of acrolein. Graph shows inhibition of the GSH peroxidase (O) and GSH S-transferase (●) activity of transferase Y_cY_{fetus} and inhibition of the GSH peroxidase (◇) and GSH S-transferase (◆) activity of transferase Y_cY_c.

TABLE I. I_{50} VALUES FOR THE INHIBITION OF THE GSH PEROXIDASE AND GSH S-TRANSFERASE ACTIVITIES OF GSH S-TRANSFERASES Y_cY_{fetus} AND Y_cY_c IN THE CASE OF BSP, ROSE BENGAL AND ACROLEIN

	Y_cY_{fetus} I_{50} (μM)		Y_cY_c I_{50} (μM)	
Inhibitor	GSH peroxidase	GSH S-transferase	GSH peroxidase	GSH S-transferase
BSP	**50**	**48**	**116**	**114**
Rose Bengal	**172**	**2,6**	**250**	**4,4**
Acrolein	**650**	**700**	**330**	**800**

Enzyme inhibition was measured with cumene hydroperoxide and CDNB as enzyme substrates.

The I_{50} values for inhibition of the GSH peroxidase activities of transferases Y_cY_{fetus} and Y_cY_c were 172 μM and 250 μM, respectively (Table I). In contrast to the peroxidase assay, the transferase activity of the two iso-enzymes was far more susceptible to inhibition, with I_{50} values of 2,6 μM and 4,4 μM for transferases Y_cY_{fetus} and Y_cY_c, respectively (Table I).

The three inhibitors were found to have no significant effects on GSH reductase.

Discussion

Analysis of the catalytic properties of the two iso-enzymes with cumene hydroperoxide as substrate showed that both iso-enzymes obey Michaelis-Menten kinetics. The results of Prohaska[13] suggested similar findings for the Y_cY_c iso-enzyme. The apparent K_m values of both iso-enzymes for GSH did not show a marked difference. However, the K_m value of the Y_cY_c iso-enzyme for cumene hydroperoxide was approximately 5 times higher than that of the fetal iso-enzyme. A low K_m can be regarded as an advantage for a substrate present in low concentrations, since this enables the enzyme concerned to catalyse the reaction rapidly. An analysis of the catalytic efficiency (K_{cat}/K_m) of the two iso-enzymes confirmed that the fetal iso-enzyme was approximately 4 times more efficient in catalysing the reduction of cumene hydroperoxide.

BSP is a substrate for the GSH S-transferases and can inhibit the activity of the transferases with other substrates.[5,7,14] The results of the effect of BSP on enzyme activity show that the ligand inhibits both the GSH peroxidase and the GSH S-transferase activity of the two iso-enzymes. Lawrence *et al.*[15] found that BSP inhibited the GSH peroxidase activity of GSH S-transferase B and the results of our study confirmed similar results for other transferases. The I_{50} values for inhibition of both GSH peroxidase and GSH S-transferase activity were almost identical for the respective iso-enzymes (Table I). This suggests that BSP is able to block the catalytic site(s) of both iso-enzymes towards both substrates in a similar manner. The fetal iso-enzyme exhibited a higher susceptibility to BSP inhibition than the Y_cY_c iso-enzyme. Alterations of the ionic conditions in the assay produced changes in the inhibition profiles of both iso-enzymes towards this substrate (data not shown). These profiles for inhibition of both the GSH peroxidase and GSH S-transferase activity of the respective iso-enzymes were similar. This suggested that ionic factors play a role in the binding of BSP to the two iso-enzymes.

The inhibition of enzyme activity by rose Bengal differed markedly from BSP inhibition, a major difference being observed between the I_{50} values obtained for GSH peroxidase inhibition and those obtained for GSH S-transferase inhibition (Table I). The GSH peroxidase activity of both iso-enzymes was approximately 60 times more resistant to inhibition than the GSH S-transferase activity. The increased susceptibility of the GSH S-transferase activity of both iso-enzymes to rose Bengal inhibition could be due to either a pH or an ionic effect. The latter seems unlikely since a high salt concentration in the incubation buffer (450 mM KCl) failed to produce major changes in the percentage inhibition (Table II). Alterations in pH have been shown to influence the binding of non-substrate ligands to the GSH S-transferases[4] and their general catalytic functions.[8,16,17] The GSH S-transferase assay was carried out at a pH of 6,5 and the GSH peroxidase assay at a pH of 7,0. Changes in pH may induce changes in the conformation of the enzyme-inhibitor complex,[7,8] which could explain to some degree the differential susceptibility to inhibition. At saturating concentrations of non-substrate ligand both GSH S-transferases Y_cY_{fetus} and Y_cY_c were able to retain approximately 40% and 50% of their GSH peroxidase activities, respectively (data not shown). Increasing the KCl concentration in the assay produced small changes in GSH peroxidase (Table III) and GSH S-transferase (Table II) inhibition of both iso-enzymes.

TABLE II. EFFECT OF KCl CONCENTRATION ON ROSE BENGAL INHIBITION OF GSH S-TRANSFERASE ACTIVITY

Iso-enzyme	Rose Bengal concentration (μM)	KCl concentration (mM)	Inhibition (%)
Y_cY_{fetus}	2	0	37
	2	450	41
Y_cY_c	4	0	47
	4	450	43

Enzyme inhibition was measured with CDNB as enzyme substrate.

I_{50} values for GSH peroxidase and GSH S-transferase activity inhibition of transferase Y_cY_{fetus} by acrolein were of a similar magnitude (Table I). The GSH peroxidase activity of the Y_cY_c iso-enzyme was, however, far more susceptible to acrolein inhibition than was the GSH S-transferase activity. The I_{50} value for GSH peroxidase inhibition was approximately

TABLE III. EFFECT OF KCl CONCENTRATION ON ROSE BENGAL INHIBITION OF GSH PEROXIDASE ACTIVITY

Iso-enzyme	Rose Bengal concentration (μM)	KCl concentration (mM)	Inhibition (%)
Y_cY_{fetus}	150	54	43
	150	270	40
Y_cY_c	150	54	38
	150	270	37

Enzyme inhibition was measured with cumene hydroperoxide as enzyme substrate.

half that obtained for the fetal iso-enzyme. In contrast, the GSH S-transferase activity of the fetal iso-enzyme was more susceptible to inhibition than the Y_cY_c iso-enzyme. This suggests that the binding of acrolein to the Y_cY_c iso-enzyme blocked the catalytic site(s) of the enzyme towards the two substrates differently. Increasing the KCl concentrations in the assays produced increases in the percentage inhibition. The effect was more marked with GSH peroxidase (Table IV) than GSH S-transferase (Table V) activity. This suggested that, like BSP, ionic factors play a role in the binding of acrolein to the two iso-enzymes.

TABLE IV. EFFECT OF KCl CONCENTRATION ON ACROLEIN INHIBITION OF GSH PEROXIDASE ACTIVITY

Iso-enzyme	Acrolein concentration (mM)	KCl concentration (mM)	Inhibition (%)
Y_cY_{fetus}	0,4	54	27
	0,4	108	33
	0,4	270	39
Y_cY_c	0,4	54	58
	0,4	108	60
	0,4	270	69

Enzyme inhibition was measured with cumene hydroperoxide as enzyme substrate.

TABLE V. EFFECT OF KCl CONCENTRATION ON ACROLEIN INHIBITION OF GSH S-TRANSFERASE ACTIVITY

Iso-enzyme	Acrolein concentration (mM)	KCl concentration (mM)	Inhibition (%)
Y_cY_{fetus}	0,3	0	25
	0,3	450	26
	0,8	0	45
	0,8	450	47
Y_cY_c	0,3	0	18
	0,3	450	21
	0,8	0	52
	0,8	450	54

Enzyme inhibition was measured with CDNB as enzyme substrate.

In summary, the results suggest that the fetal iso-enzyme is far more efficient than GSH S-transferase Y_cY_c in catalysing the reduction of organic peroxides. At a pH of 7,0, which is also the normal intracellular pH,[18] the GSH peroxidase activity of both iso-enzymes can be inhibited by BSP binding. The fetal iso-enzyme was able to retain some degree of GSH

peroxidase activity despite saturating non-substrate ligand concentrations of rose Bengal. This may be physiologically important in certain toxicological and pathological states where concentrations of non-substrate ligands are high and to which the fetus may be exposed *in utero*. The GSH peroxidase activity of the fetal iso-enzyme was far less susceptible to acrolein inhibition than the Y_cY_c iso-enzyme. It is therefore attractive to suggest that, should a similar situation arise *in vivo*, this resistance to peroxidase inhibition may play a role in preventing the fetotoxic effects of acrolein.

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