Detoxification of DNA hydroperoxide by glutathione transferases and the purification and characterization of glutathione transferases of the rat liver nucleus

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DNA peroxidized by exposure to ionizing radiation in the presence of oxygen is a substrate for the Se-independent GSH peroxidase activity of several GSH transferases, GSH transferases 5–5, 3–3 and 4–4 being the most active in the rat liver soluble supernatant fraction (500, 35 and 20 nmol/min per mg of protein respectively) and GSH transferases μ and π the most active, so far found, in the human liver soluble supernatant fraction (80 and 10 nmol/min per mg respectively). Although the GSH transferase content of the rat nucleus was found to be much lower than that of the soluble supernatant, nuclear GSH transferases are likely to be more important in the detoxification of DNA hydroperoxide produced *in vivo*. Two nuclear fractions were studied, one extracted with 0.075 M-saline/0.025 M-EDTA, pH 8.0, and the other extracted from the residue with 8.5 M-urea. The saline/EDTA fraction contained subunits 1, 2, 3, 4 and a novel subunit, similar but not identical to 5, provisionally referred to as 5*, in the proportions 40:25:5:5:25 respectively. The 8.5 M-urea-extracted fraction contained principally subunit 5* together with a small amount of subunit 6 in the proportion 95:5 respectively. GSH transferase 5*–5* purified from the 8.5 M-urea extract has the highest activity towards DNA hydroperoxide of any GSH transferase so far studied (1.5 μ mol/min per mg). A Se-dependent GSH peroxidase fraction from rat liver was also active towards DNA hydroperoxide; however, since this enzyme accounts for only 14% of the GSH peroxidase activity detectable in the nucleus, GSH transferases may be the more important source of this activity. The possible role of GSH transferases, in particular GSH transferase 5*–5*, in DNA repair is discussed.

INTRODUCTION

Free radicals in the presence of oxygen have a number of toxic effects in the cell, including the initiation of lipid peroxidation (Pryor, 1976) and the induction of potentially mutagenic lesions in DNA (Ames & Saul, 1985). A number of systems exist which might protect against such radical damage, including Se-dependent GSH peroxidase and Se-independent GSH peroxidases, i.e. GSH transferases. For example, these enzymes might detoxify and repair free radical damage to membrane lipid. They have been shown to inhibit microsomal lipid peroxidation by reducing free fatty acid hydroperoxides released from peroxidized membrane by phospholipase A (Grossmann & Wendel, 1983; Sevanian *et al.*, 1983; Tan *et al.*, 1983, 1984; Meyer *et al.*, 1985b) and van Kuijk *et al.* (1986) have suggested that such excision and detoxification steps are followed by repair of the membrane by lysophosphatide acyl-CoA transferase.

Evidence is accumulating that GSH peroxidase activity might also participate in the detoxification and repair of radical damage to DNA. For example, Christopherson (1969) showed that rat liver soluble supernatant has GSH peroxidase activity towards both 5-hydroperoxymethyl uracil and DNA exposed to high energy irradiation in the presence of oxygen (DNA hydroperoxide) and Tan *et al.* (1986) showed that both Se-

dependent GSH peroxidase and purified GSH transferases from the soluble supernatant catalyse the reduction of 5-hydroperoxymethyluracil. In the present paper we investigate the activities of Se-dependent GSH peroxidase and purified GSH transferases towards DNA hydroperoxide and demonstrate the presence in the nucleus of a novel GSH transferase which is more active than other GSH transferases towards DNA hydroperoxide. A possible role for nuclear GSH transferases in DNA repair is discussed. Part of this work has been reported in a previous communication (Tan *et al.*, 1987a).

METHODS

Preparation of DNA hydroperoxide

DNA hydroperoxide was prepared by a modification of the method of Daniels *et al.* (1957). Calf thymus DNA (150 mg, sodium salt, type I; Sigma, Poole, Dorset, U.K.) in 100 ml of h.p.l.c.-grade water was irradiated for 75 min at 2 krad per min at 26 °C using a Maximar 250 kVp X-ray machine and freeze-dried to remove H₂O₂ generated during irradiation. The resulting DNA hydroperoxide was dissolved in 4 ml of h.p.l.c.-grade water and the DNA-bound peroxide content found to be 0.12 mM by the spectrophotometric assay of Buege & Aust (1978).

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Preparation of enzymes and measurement of enzyme activities

GSH transferase isoenzymes were isolated from the soluble supernatant fraction of rat liver, kidney and testis by methods based on those of Beale *et al.* (1983) and Meyer *et al.* (1985a), and from human liver as described by Ostlund Farrants *et al.* (1987). Se-dependent GSH peroxidase and GSH transferase fractions were prepared according to Tan *et al.* (1986). Nuclei were prepared by homogenization of rat liver in 0.05% (w/v) citric acid followed by centrifugation twice through 2.3 M-sucrose exactly according to Taylor *et al.* (1973). Nuclei prepared this way have been shown to be free from contamination (Taylor *et al.*, 1973).

Two GSH transferase-containing fractions were extracted from freshly prepared nuclei, one extracted with 0.075 M-NaCl/0.025 M-EDTA, pH 8.0, the other extracted from the nuclear residue with 8.5 M-urea. The urea extract was renatured over 48 h by dialysis against decreasing concentrations of urea (4, 2, 1, 0.5 and 0.25 M) followed by 0.03 M-sodium phosphate, pH 7.0. These dialyses restored enzymic activity to the fraction. The GSH transferases from both the saline/EDTA and 8.5 M-urea fractions were separated from other proteins by GSH agarose affinity chromatography according to Vander Jagt *et al.* (1985) and analysed by isoelectric focusing (Beale *et al.*, 1983); f.p.l.c. chromatofocusing on a mono P column (Pharmacia, Uppsala, Sweden) equilibrated with 25 mM-diethanolamine/HCl, pH 9.6 and eluted with Polybuffer 96/HCl, pH 6.0; reverse-phase h.p.l.c. according to the method of Ostlund-Farrants *et al.* (1987), and SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

GSH transferase and peroxidase activities were assayed according to Habig *et al.* (1974) and Prohaska & Ganther (1976) respectively. The concentration of DNA hydroperoxide used as substrate was 30 μ M.

Immunological studies

Immunological characterization of GSH transferases was carried out by both double immunodiffusion and Western blotting (Towbin *et al.*, 1979) using antisera to rat GSH transferases 1-2 and 3-4 raised in rabbits.

RESULTS

DNA hydroperoxide as a substrate for Se-dependent GSH peroxidase and GSH transferase isoenzymes

Table 1 shows that DNA hydroperoxide is a substrate for both Se-dependent GSH peroxidase and GSH transferases of rat liver soluble supernatant. It is a good substrate for GSH transferases 5-5, 4-4 and 3-3, but a poor substrate for GSH transferases 6-6, 7-7 and 2-2. Human GSH transferases are comparable in that GSH transferase μ , which is similar to rat GSH transferases 3-3 and 4-4, is the most active isoenzyme.

The saline/EDTA-extractable GSH transferase fraction from rat liver nuclei

The saline/EDTA extractable GSH transferases obtained from the GSH affinity column were separated by isoelectric focusing which showed the presence of all the isoenzymes found in the soluble supernatant, but in different relative amounts (Fig. 1). In particular, there was a marked enhancement in a component with the

Table 1. DNA hydroperoxide as a substrate for the GSH transferases and the Se-dependent GSH peroxidase

n.d., not determined.

Enzyme	Class	Specific activity (nmol/min per mg of protein)
GSH transferase		
Rat	1-1	α
	2-2	α
	3-3	μ
	4-4	μ
	5-5	n.d.
	5*-5*	n.d.
	6-6	μ
Human	7-7	π
	α - ϵ	α
	μ	μ
Rat Se-dependent GSH peroxidase fraction	π	π
		20.0

properties of GSH transferase 5-5, namely a pI of 7.0, high activity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane and cumene hydroperoxide, low activity towards 1-chloro-2,4-dinitrobenzene and the same subunit molecular mass on SDS/polyacrylamide-gel electrophoresis,

Fig. 1. Analysis of saline/EDTA extract of nuclei by isoelectric focusing

A saline/EDTA extract of nuclei obtained from 90 g of liver was dialysed and subjected to isoelectric focusing in the pH range 5-10 as described in the text. Fractions were assayed for GSH transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and 1,2-dichloro-4-nitrobenzene (results not shown) and for GSH peroxidase activity towards cumene hydroperoxide (Cu-OOH) as described in the text.

Fig. 2. Analysis of nuclear GSH transferases by reverse-phase h.p.l.c.

Native GSH transferases obtained by affinity chromatography were analysed by reverse-phase h.p.l.c. as described in the text. GSH transferases of the soluble supernatant (trace *a*) are compared with those of the saline/EDTA extract (trace *b*) and the urea extract (trace *c*). Samples are equivalent to 0.01, 3 and 10 g of liver respectively.

i.e. equivalent to subunits 3 and 4 or Yb (Meyer *et al.*, 1984). However, this component is not identical to GSH transferase 5–5 since, unlike GSH transferase 5–5, it is adsorbed to GSH agarose. Because of its similarity to GSH transferase 5–5 it is provisionally called GSH transferase 5*–5*. The subunit composition of the saline/EDTA-extracted GSH transferases was quantified by reverse-phase h.p.l.c. (Fig. 2, Table 2). In this separation subunits 2 and 5* have the same retention time and were shown to be present in approximately equal amounts by analysis of the h.p.l.c. fraction by SDS/polyacrylamide-gel electrophoresis.

The urea-extracted GSH transferase fraction from rat liver nuclei

When the GSH transferase fraction from the 8.5 M-urea extract was separated by f.p.l.c. chromatofocusing a major and a minor peak were obtained (Fig. 3). The

Table 2. The composition of GSH transferases in rat liver cytosol and nucleus

GSH transferase subunits were separated using reverse-phase h.p.l.c. (Ostlund Farrants *et al.*, 1987) and nuclear and cytosol concentrations calculated utilizing the hepatocyte compartment structure deduced by Drabkin (1975) in which the cytosol and nucleus occupy 60% and 8% respectively of the hepatocyte volume. n.o. = not observed.

GSH transferase subunit	GSH transferase content		
	Nucleus ($\mu\text{g/ml}$ of nucleus)		Cytosol ($\mu\text{g/ml}$ of cytosol)
	Saline/EDTA extract	Urea extract	
1	28	n.o.	4033
2	17	n.o.	1013
3	4	n.o.	1063
4	4	n.o.	1833
5	n.o.†	n.o.	24
5*	16	13	n.o.
6	n.o.	1	150
7	< 1	n.o.	67

† The absence of subunit 5 in the nucleus and subunit 5* in the soluble supernatant fraction has not been totally excluded.

major peak eluting at pH 7.8 had activity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane and DNA hydroperoxide, but little activity towards 1-chloro-2,4-dinitrobenzene. It had the same retention time on reverse-phase h.p.l.c. and the same mobility on SDS/polyacrylamide-gel electrophoresis as GSH transferases 5–5, but because of its affinity for GSH agarose is believed to be GSH transferase 5*–5*. The activity towards DNA hydroperoxide of GSH transferase 5*–5* purified from 8.5 M-urea extracts is 1.5 $\mu\text{mol/min}$ per mg of protein, which is even higher than that of GSH transferase 5–5 from the soluble supernatant fraction. The minor peak eluting at pH 7.5 was active only towards 1-chloro-2,4-dinitrobenzene and had the same retention time on reverse-phase h.p.l.c. and mobility on SDS/polyacrylamide-gel electrophoresis as GSH transferase 6–6. This isoenzyme composition was confirmed by reverse-phase h.p.l.c. which showed the separation of this fraction into a major peak containing subunit 5* and a small shoulder containing subunit 6 (Fig. 2c and Table 2).

The GSH transferase 5*–5* fraction from urea extracts was tested with antisera against GSH transferases 1–2 and 3–4 using double immunodiffusion and Western blotting, but no cross-reactivity was observed, indicating that subunit 5* is unlikely to be a member of either the Alpha or Mu GSH transferase multigene families (Taylor *et al.*, 1987).

Relative contribution of Se-independent and Se-dependent GSH peroxidases in the nucleus

The contributions of Se-independent (GSH transferases) and Se-dependent GSH peroxidases to total GSH peroxidase activity in the rat liver soluble supernatant fraction using cumene hydroperoxide as substrate

Fig. 3. Analysis of the urea extract of nuclei by f.p.l.c. chromatofocusing

The GSH transferase fraction obtained by affinity chromatography of a urea extract of saline/EDTA extracted nuclei (equivalent to 80 g of liver) as described in the text was transferred into 25 mM-diethanolamine/HCl, pH 9.6, and applied to a Mono P column equilibrated in this buffer. The column was eluted with Polybuffer 96 adjusted to pH 6 with HCl. Fractions were assayed for GSH transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and GSH peroxidase activity towards peroxidized DNA (DNA-OOH).

were 4.3 and 9.2 $\mu\text{mol}/\text{min}$ per ml of cytosol respectively, and in the saline/EDTA extracted fraction from the nucleus were 7.5 and 1.25 nmol/min per ml of nuclei respectively. Thus whereas GSH transferases account for only 32% of the total GSH peroxidase in the soluble supernatant fraction, in the soluble fraction of the nucleus they account for 86%. No Se-dependent GSH peroxidase activity was recovered in the urea-extracted fraction.

DISCUSSION

Christopherson (1969) showed that thymine and DNA which had been peroxidized by X-irradiation in the presence of oxygen oxidized GSH to GSSG and that this reaction was catalysed by the rat liver soluble supernatant fraction. This catalysis was assumed to be due to GSH peroxidase activity. Recently we extended this work, showing that the thymine hydroperoxide, 5-hydro-

peroxymethyluracil, was a substrate, not only for Se-dependent GSH peroxidase, but also certain GSH transferase isoenzymes (Tan *et al.*, 1986). In the present paper we show that DNA hydroperoxide is also a substrate for both classes of GSH peroxidase and that the specificity of GSH transferase isoenzymes for the macromolecular hydroperoxide substrate is quite different from that towards free 5-hydroperoxymethyluracil. Thus, while GSH transferases 1-1, 2-2, 6-6 and 7-7 are good enzymes for the free thymine hydroperoxide, GSH transferases 3-3, 4-4, and in particular 5-5, are good enzymes for DNA hydroperoxide. In addition to 5-hydroperoxymethyluracil, DNA also contains two other thymine hydroperoxides, namely 5-hydroperoxy-6-hydroxy-5,6-dihydrothymine and 5-hydroxy-6-hydroperoxy-5,6-dihydrothymine (Schulte-Frohlinde & von Sonntag, 1985), the enzyme specificities of which are not known. It is not known whether the presence of these other thymine hydroperoxides determines the apparently different utilization of DNA hydroperoxide by GSH transferases or whether the difference in utilization is related to the macromolecular double-stranded polynucleotide structure of the DNA.

If either the GSH transferases or the Se-dependent GSH peroxidase are to be important for the reduction of DNA hydroperoxides *in vivo* it is important that they should have a nuclear location, accessible to DNA. We have shown that both GSH transferase and Se-dependent GSH peroxidase are present in the rat liver nucleus, but that the GSH transferases make the major contribution to total GSH peroxidase activity. These nuclear GSH transferases have been studied in two fractions: one extracted by saline/EDTA and the other extracted from the saline/EDTA residue by 8.5 M-urea. The saline/EDTA-extracted fraction contains all the GSH transferases present in the soluble supernatant fraction, but in different relative amounts and in addition GSH transferase 5*-5* which, although it resembles GSH transferase 5-5 in its enzymic activity and many of its physical properties, differs in being adsorbed to GSH agarose. The 8.5 M-urea-extracted fraction is particularly interesting, since, being more firmly bound to chromatin, it is more likely to participate in detoxification reactions involving DNA. We have been able to study this fraction in some detail because of the ease with which its GSH transferase components renature after removal of urea by dialysis. It has a major component which is GSH transferase 5*-5* and a minor component which is GSH transferase 6-6. GSH transferase 5*-5* isolated from the nucleus is even more active towards DNA hydroperoxide than GSH transferase 5-5 from the cytoplasm. GSH transferase 5*-5* would appear to be a novel GSH transferase.

The only other study relating to the isolation of nuclear GSH transferases is that of Bennett *et al.* (1986), who observed that a non-histone protein, BA, has an amino acid content similar to that of the GSH transferases and an apparent M_r on SDS/polyacrylamide gels similar to GSH transferase subunits 3, 4 and 5 (Jakoby *et al.*, 1984; Meyer *et al.*, 1984). The relationship between GSH transferases 5*-5*, 5-5 and non-histone protein BA is under investigation.

The results described in this paper suggest a possible role for GSH transferases such as 5*-5* as part of a repair system *in vivo*. If analyses made on DNA in the presence of oxygen *in vitro* are a guide, the most likely

substrates for such GSH transferases in DNA damaged by free radical attack *in vivo* are thymine hydroperoxide residues (Schulte-Frohlinde & von Sonntag, 1985). Since these hydroperoxides are decomposed by metal ions to radicals which are mutagenic (Thomas *et al.*, 1976) GSH transferases provide a means for their detoxification. The thymine glycol and 5-hydroxymethyluracil residues which result, although no longer chemically reactive, are cytotoxic and cytostatic, apparently preventing DNA replication (Meldrum *et al.*, 1974; Waschke *et al.*, 1975) and therefore must be removed. Recently, thymine glycol DNA glycosylase and 5-hydroxymethyluracil DNA glycosylase, enzymes which remove these cytostatic pyrimidine residues, have been identified (Ames & Saul, 1985; Hollstein *et al.*, 1984). DNA repair is presumably completed by the successive action of endonuclease, DNA polymerase and DNA ligase activity and the thymine derivatives released are excreted in the urine. Ames & Saul (1985) have demonstrated the presence of thymine glycol and 5-hydroxymethyl uracil as normal constituents of urine, their occurrence in an organism being proportional to its rate of oxygen utilization. In the present study we have obtained peroxidized DNA *in vitro* using high energy irradiation; however, it is assumed that DNA *in vivo* is accessible to active oxygen species which arise as by-products of oxygen metabolism, namely either primary oxygen radicals or secondary radicals such as products of lipid peroxidation (Tan *et al.*, 1987b).

The overall GSH transferase content of the nucleus determined in the present study is about three orders of magnitude lower than that of the soluble cytoplasm which raises two questions. Firstly, are the nuclear enzymes merely cytoplasmic contaminants and secondly, is the nuclear enzyme concentration high enough to be effective as a DNA repair enzyme? In answer to the first question, the GSH transferase composition of the nucleus is quite different from that of the soluble cytoplasm, GSH transferase 5*-5*, an isoenzyme which has yet to be detected in the soluble cytoplasm, being a major component. With respect to the second question, the concentration of GSH transferase 5*-5*, although low compared with GSH transferases in the soluble cytoplasm, corresponds to about 140000 molecules of enzyme per nucleus, which is comparable with a DNA repair enzyme such as O⁶-alkylguanine-DNA alkyltransferase which occurs at a concentration of about 70000 molecules of enzyme per nucleus (Lindahl, 1982). Thus the results we have obtained are consistent with the role of GSH transferase 5*-5* as a DNA repair enzyme.

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