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SELENIUM- AND NON-SELENIUM-DEPENDENT GLUTATHIONE PEROXIDASES IN MOUSE LIVER

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Abstract—About 70% of the activity of mouse liver peroxidase utilizing H_2O_2 as well as *t*-butyl hydroperoxide as substrates is the selenium-dependent glutathione peroxidase. With cumene hydroperoxide as substrate the sum of non-Se-dependent and Se-dependent enzymes has been measured. Se-deficient mice have about 30% of the total activity of Se-adequate mice, with cumene hydroperoxide as substrate. Glutathione *S*-transferase activity was not affected by Se deficiency. Fractionation on DEAE-Sepharose and subsequently on CM-Sepharose showed at least four transferase activities, and besides the Se-GSH-Px, two non-Se-GSH-Px activities detectable with cumene hydroperoxide that did not coincide with any of the transferases. For the major activity the apparent Michaelis constants at 1 mmole/l GSH were found to be 0.07 mM for linoleic acid hydroperoxide, 10 mM for *t*-butyl hydroperoxide, and 0.11 mM for cumene hydroperoxide. The ratios of the apparent maximum velocities of these hydroperoxides were 1 (linoleic):100 (butyl):3000 (cumene). This has the practical consequence that the cumene hydroperoxide-supported reaction is extremely favored under standard assay conditions. The results suggest that data based on only a single organic peroxide and hydrogen peroxide can lead to invalid conclusions as to the nature of the enzymes that are capable of catalyzing GSH-linked reduction of hydroperoxides.

Subcellular fractionation showed that cytosol, mitochondria and microsomes contain both types of peroxidase, although in different proportions. The distribution as well as the kinetic properties suggest the Se-GSH-Px is exceedingly faster in reducing organic, including fatty acid, hydroperoxides.

In addition to selenium-dependent glutathione peroxidase (EC 1.11.1.9) a non-Se-GSH-Px† activity has been discovered in livers of Se-deficient rats [1], an activity that is also exhibited by rat liver glutathione S-transferase B [2, 3]. Detailed studies of substrate specificity with Se-GSH-Px from bovine red blood cells [cf. Ref. 4] have not been performed with non-Se-GSH-Px. Recently, a GSH-Px-like activity in mouse heart and liver was found active with cumene hydroperoxide as well as with H_2O_2 [5]. This mitochondrial activity was reported to be unaffected in Se deficiency and differed from the GSH S-transferase-related non-Se-GSH-Px activity.

The recent discussion of the necessity and value of dietary Se supplements for man [6, 7] shows that our knowledge of the contribution of Se- and non-Se-Px to cellular protection is not clear. However, the first information needed for the molecular understanding of the role of the enzymes, the quantitative determination of the enzymes in various organs of different species, is unavailable. Conflicting data on the proportion of Se- and non-Se-GSH-Px in different species (rat, hamster, guinea pig and rabbit) led us to investigate the relative amounts of these enzymes in mice. It was also of interest whether in a rodent closely related to the rat, the mouse, non-Se-GSH-Px activity is also a part of GSH *S*-transferase. This work shows that the mouse liver enzyme has different properties and that findings obtained for the rat do not apply to other species.

MATERIALS AND METHODS

The livers of 15 mice were homogenized in 0.1 mole/l potassium phosphate at pH 7.4 in the presence of 1 mmole/l EDTA (1 ml buffer per g liver wet wt). Twenty-six and a half ml of the 100,000 *g* supernatant were dialyzed against 10 mmole/l Tris–HCl at pH 8.0 and applied to a column (2.5 × 14 cm) of DEAE-Sepharose equilibrated with the same buffer. The column was eluted with a linear salt gradient over 135 fractions from 0 to 1 mole/l KCl. Fractions of 6.5 ml were collected. The eluate of the DEAE-column which showed GSH *S*-transferase as well as GSH-Px activity was rechromatographed on a CM-Sepharose column (1 × 92 cm) equilibrated with 10 mmole/l potassium phosphate, pH 6.7, washed with the same buffer and eluted with a gradient from 0–75 mmole/l KCl, then 75–1000 mmole/l KCl as indicated.

Mice were fed for at least 50 days with a Se^-/Se^+ diet: 30% torula yeast, 61% sucrose, 3% soybean oil, 5% salt and 1% vitamin mix, provided by Dr. R. F. Burk, San Antonio, TX. The Se^+ diet was supplemented with 0.5 ppm Se as Na_2SeO_3.

Glutathione peroxidase activity was assayed at 37° by the coupled test procedure using GSH reductase/NADPH and 1.25 mmole/l hydroperoxide as indicated [8, 9]. Glutathione *S*-transferase activity

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† Abbreviations used: CuOOH, cumene hydroperoxide; BuOOH, *t*-butyl hydroperoxide; Se-GSH-Px, selenium-dependent glutathione peroxidase; non-Se-GSH-Px, non-selenium-dependent glutathione peroxidase; Se^-/Se^+, selenium deficient/supplemented; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

was assayed [10] at 25° with 1-chloro-2,4-dinitrobenzene (CDNB) or with 1,2-dichloro-4-nitrobenzene (DCNB). Protein was determined according to Lowry's procedure with bovine serum albumin (Sigma) as the standard. Glutathione was from Waldhof-Pharma, Aschaffenburg, West Germany; NADPH and glutathione reductase from Boehringer, Mannheim; *t*-butyl hydroperoxide, ethyl hydroperoxide and cumene hydroperoxide from Peroxid-Chemie, Höllriegelskreuth, West Germany. The purity of CuOOH was checked by high-performance liquid chromatography. The gradient and the isocratic system described in [14] were used. Commercial CuOOH contained four additional components including acetophenone. Purified GSH-Px prepared according to [9] as well as partially purified non-Se-GSH-Px from mouse liver (this work) reacted with the fraction corresponding to CuOOH. Linoleic and linolenic acid hydroperoxide were prepared by oxidizing commercially available fatty acids in 0.1 mmole/l Na-borate buffer (pH 9.0) in the presence of soybean lipoxygenase (Sigma). The products were acidified with 5 mole/1 HCl, extracted twice with ethyl ether, evaporated and dissolved in methanol.

For subcellular fractionation, a homogenate was prepared containing 2.5 mmole/l HEPES at pH 7.5, 220 mmole/l mannitol, 70 mmole/l sucrose and 1 mmole/l EDTA. The nuclear fraction was sedimented after 3 min at 900 *g*. Mitochondria were sedimented after 5 min at 12,000 *g*, resuspended twice and recentrifuged at 8500 *g* for 10 min in 145 mmole/l KCl, 2.5 mmole/l HEPES at pH 7.5. The mitochondrial pellet was resuspended in the same medium containing 0.5% Tritox X-100 and the enzymes were measured. Post mitochondrial supernatant was centrifuged for 35 min at 105,000 *g*, the microsomes were washed once and finally suspended in 50 mmole/l Tris–HCl, pH 7.4, containing 20% glycerol and 0.5% Triton X-100.

Kinetic experiments were performed as described [8] with the exception that additional GSH reductase was used (15 μg/ml, Boehringer) to overcome inhibition of the enzyme by CuOOH at concentrations above 1 mmole/l. Lactate dehydrogenase and glutamate dehydrogenase were determined as described [11] in the presence of 0.2 nmole rotenon per mg of protein and 0.2 nmole antimycin A per mg of protein.

RESULTS AND DISCUSSIONS

When assayed with H_2O_2 or BuOOH as substrates, liver homogenates of Se^- mice exhibited less than 5% of the GSH-Px activity of control animals. With CuOOH as substrate a higher activity than with H_2O_2 or BuOOH was found in both groups (Table 1). The difference between the Se^+ and Se^- groups, however, remained constant. This indicates that a part of the CuOOH-dependent activity (~30% of the total activity) is not related to the Se status. However, GSH *S*-transferase activity from Se^- mouse liver homogenates were not significantly different from controls at this time after the onset of Se deficiency.

To discriminate among the several GSH-Px activities, mouse liver cytosol was fractionated on a column of DEAE-Sepharose and the eluate assayed for GSH-Px and GSH *S*-transferase activities (Fig. 1). At least two major peaks of activity were observed with each of the hydroperoxides. The first peak fraction exhibited high GSH-Px activity with CuOOH and little activity with either H_2O_2 or BuOOH. In contrast, identical enzymatic rates were measured with all three hydroperoxides in the second fraction. Thus, the second peak fraction represents Se-GSH-Px. With respect to H_2O_2 as substrate, our results obtained with mouse liver confirm previous work with the rat [12]. However, the elution pattern in Fig. 1 indicates that, if assayed with *t*-butyl hydroperoxide, the non-Se-GSH-Px escapes attention. Also, the pattern raises doubts as to the coelution of non-Se-GSH-Px and GSH *S*-transferase B reported for the rat. Therefore, we rechromatographed the GSH *S*-transferase fractions on CM-Sepharose. At least four major transferases activities were separated (Fig. 2).

The GSH *S*-transferase and non-Se-GSH-Px activity profiles do not coincide strictly. The peak of one enzyme activity is accompanied by a shoulder in the elution profile of the other (and vice versa) which may indicate that enzymes catalyze both reactions, although with different rates. Similar complex relationships were reported for guinea pig liver [13].

An orientating study of the kinetic parameters of three hydroperoxides was performed with the second peak fraction (Fig. 2, upper panel, designated as non-Se-GSH-Px). At the fixed substrate concentrations used (1.25 mmole/1 ROOH, 1 mM GSH), this non-Se-GSH-Px fraction exhibited less than 1.5% activity compared to CuOOH with the following hydroperoxides: H_2O_2, ethyl-, and linolenic acid hydroperoxide. With three of the hydroperoxides showing a measurable reaction, initial rate kinetics were done at a fixed cosubstrate concentration of 1 mmole/l GSH. Table 2 shows that the apparent

Table 1. Specific activities of mouse liver GSH peroxidases and GSH *S*-transferases from Se-deficient and Se-supplemented animals assayed with a number of substrates

	Glutathione peroxidases			Glutathione *S*-transferases	
	H_2O_2 (*n*)	BuOOH (*n*)	CuOOH (*n*)	CDNB (*n*)	Ethacrynic acid (*n*)
Se^-	14 ± 6 (10)	14 ± 5 (10)	140 ± 20 (10)	1400 ± 300 (7)	87 ± 15 (9)
Se^+	320 ± 50 (8)	320 ± 60 (8)	480 ± 70 (8)	1460 ± 370 (7)	63 ± 24 (9)

Data are given in mU/mg protein (according to Ref. 9 for GSH-Px and Ref. 10 for GSH *S*-transferase) ± S.D. (*n* = number of animals).

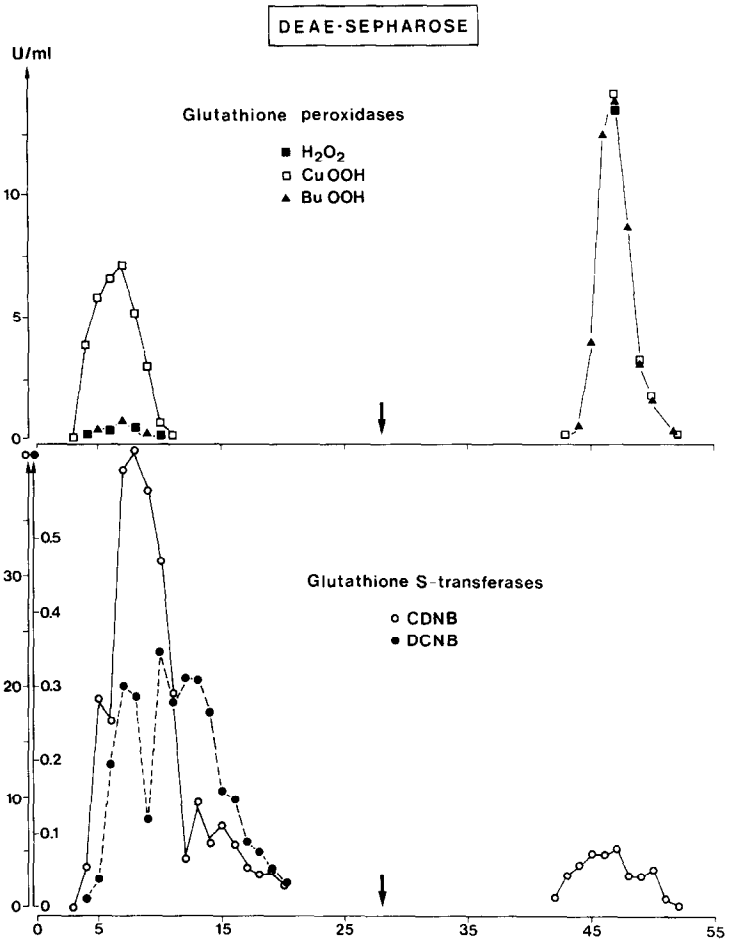

Fig. 1. Elution pattern for GSH-Px and GSH *S*-transferase activity of mouse liver cytosol upon chromatography on DEAE-Sepharose.

maximum velocities differ by orders of magnitude. This data explains why CuOOH and BuOOH yield such different enzymatic activities in the standard assay: BuOOH is assayed below K_m. The actual enzymic rate at physiological hydroperoxide concentration ($<10^{-6}$ M) is proportional to the quotient $\frac{V_{max}}{K_m}$, which for linolenic acid hydroperoxide, for example, is smaller than for CuOOH by a factor of 2300. This consideration raises serious doubts as to the physiological function and normal substrate of the enzyme from mouse liver.

Table 2. Apparent kinetic parameters of mouse liver non-Se-GSH-Px for three different organic hydroperoxides at one fixed cosubstrate concentration.

	K_m (app)*	V_{max} (app)†
BuOOH	10	0.49
CuOOH	0.11	15
Linoleic OOH	0.074	0.005

Conditions: 50 mmole/l potassium phosphate at pH 7.0; 1 mmole/l GSH. The data were computed by a weighted linear regression fit of the double reciprocal plot according to Wilkinson [21].

* mmole/l.

† μmole/min/ml assay.

Figure 2 indicates that another minor GSH-Px-like activity is eluted from CM-Sepharose with the wash. This activity was also partially characterized by initial rate measurements. It did not react with H_2O_2. The K_m (app) for BuOOH was 50 μmole/l, and that for CuOOH 40 μmole/l. The finding suggests the presence of two separate non-Se-GSH-Px activites. To compare the activities with a Se-independent, membrane-bound GSH-Px of mouse liver and heart [5], subcellular fractionation was performed yielding the data in Table 3. The results differ from the findings of Katki and Myers [5] by orders of magnitude in both the observed specific activities and in the subcellular distribution. To aid interpretation of this data, concentration as well as the total content of each of the enzymes was calculated for the compartments based on the following: 4.8 μl/mg total cytosolic water and 3.8 μl/mg osmotic cytosolic water; 1.6 μl/mg total mitochondrial and 0.8 μl/mg osmotic mitochondrial water; 80 mg cytosolic, 50 mg mitochondrial, 30 mg microsomal protein per g liver wet wt [15]. The following conclusions are drawn:

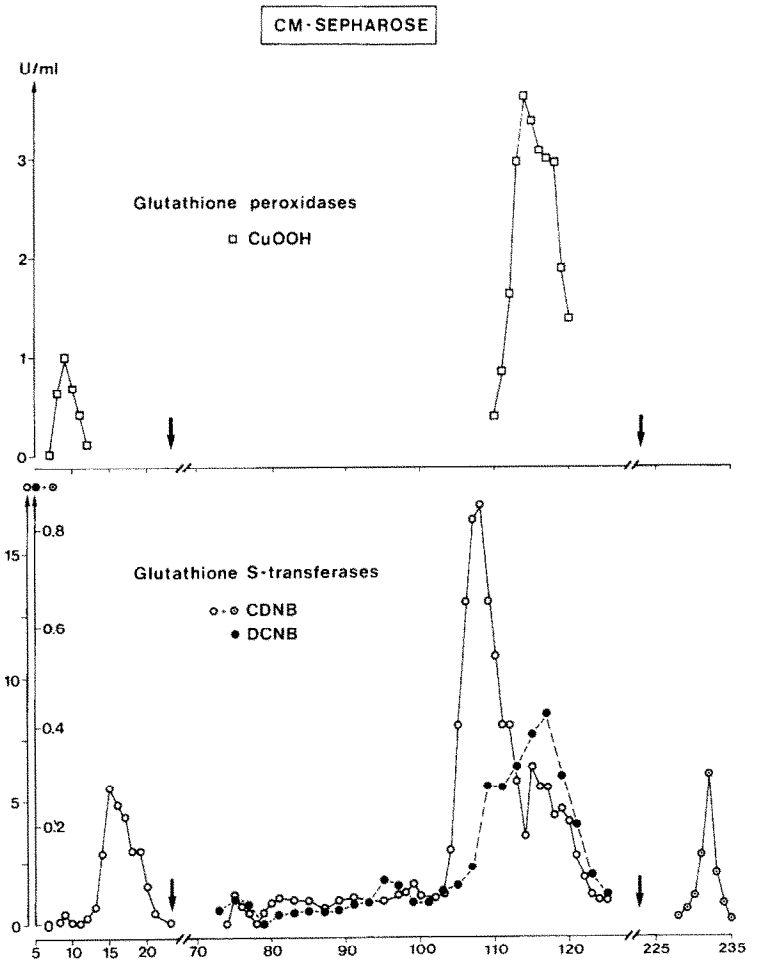

Fig. 2. Elution pattern for GSH-Px and GSH *S*-transferase activity upon rechromatography on CM-Sepharose of fractions 4–16 (Fig. 1).

1. The differences with and without selenium in the diet are nearly equal for CuOOH- and H_2O_2-reactive GSH-Px in cytosol and microsomes (and insignificant although possible for mitochondria). The activity assayed with H_2O_2 as substrate represents exclusively the Se-dependent activity, whereas that assayed with CuOOH is the sum of Se-dependent and Se-independent activities.

2. Cytosol and mitochondria contain 75 and 25%, respectively, of the Se-GSH-Px. The concentration of the enzyme in mitochondria is 50% fold greater than in cytosol. Mitochondria contain ~20% of the non-Se-GSH-Px; its *concentration* in the cytosol, however, is lower by a factor of 3 than in mitochondria.

3. The ratio of Se-Px to non-Se-Px content is about 2:1 in the cytosol, approximately 1:10 in mitochondria.

4. Compared to the calculated content of lactate dehydrogenase contamination, mouse liver mitochondria seem to lack GSH *S*-aryltransferase activity independent of their selenium status. The presence of this enzyme in this organelle has been clearly demonstrated for the rat [16, 17].

5. Because the water space of the smooth endoplasmic reticulum is unknown the microsomal content can only be crudely estimated. However, mouse liver microsomes do contain Se- as well as non-Se-GSH-Px activity, and GSH *S*-transferases (as described for rat liver [18–20]).

The results of cell fractionation support the assumption that mouse liver GSH *S*-transferase differ considerably from those of other species with respect to distribution as well as kinetic properties. Considering localization, concentration and enzymatic turnover, the data suggest that, in the mouse, Se-GSH-Px plays the dominant role in reducing organic hydroperoxides.

The work reported here suggests that the increasing complexity of the field of GSH peroxidases may be due in part to methological pitfalls. It does not seem possible to differentiate the Se-dependent and the Se-independent GSH-Px in tissue homogenates of organs by using a single organic hydroperoxide and H_2O_2 as the substrates for establishing this difference.

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Table 3. Subcellular distribution and sensitivity to dietary selenium of mouse liver GSH peroxidases, GSH *S*-aryltransferase and marker enzymes

Activity	Cytosol		Mitochondria		Microsomes	
	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+
GSH-Px with H_2O_2*	3.8 ± 2.6	642 ± 49	0.6 ± 0.5	200 ± 27	n.d.	15 ± 5
GSH-Px with CuOOH*	310 ± 24	1000 ± 40	21 ± 3.7	199 ± 28	26 ± 4	40 ± 5
GSH *S*-aryltransferase*	2410 ± 677	2650 ± 597	44 ± 3	40 ± 8	122 ± 16	153 ± 37
Lactate dehydrogenase†	2.67 ± 0.08	3.0 ± 0.04	0.05 ± 0.002	0.05 ± 0.007	0.03 ± 0.008	0.04 ± 0.016
Glutamate†	0.034 ± 0.006	0.038 ± 0.003	2.0 ± 0.26	1.93 ± 0.35	0.011 ± 0.003	0.023 ± 0.006

* mU/mg protein.
† U/mg protein.
n.d. = not detectable.
$n = 3$.

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Note added in proof—After a 6-month period on the Se^- diet, GSH transferase activity assayed by CDNB was 2.1-fold, and assayed by DCNB 3.2-fold times the Se^+ control. Obviously existing long-term effects of Se deficiency need separate consideration.

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