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Identification of a hepatic plasma membrane glutathione *S*-transferase activated by *N*-ethylmaleimide

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Rat liver plasma membranes exhibit membrane-bound glutathione *S*-transferase actvity. The specific activity in isolated canalicular membranes was 83 ± 8 mU/mg protein and 50 ± 3 mU/mg protein in the sinusoidal membranes. Whereas microsomal and outer mitochondrial glutathione *S*-transferases were stimulated seven and four-fold with *N*-ethylmaleimide, respectively, the plasma membrane activity was activated two-fold. Western blot analysis, using an antibody against the microsomal glutathione *S*-transferase, shows the presence of a 17 kDa protein in canalicular and sinusoidal membrane fractions. The antibody reaction was about three-fold higher in the canalicular compared to the sinusoidal membrane fraction. These data support the conclusion that the plasma membrane glutathione *S*-transferase is closely related to the microsomal and outer mitochondrial membrane enzyme.

Introduction

Membrane-bound GSH *S*-transferase has been found in the endoplasmic reticulum and outer mitochondrial membrane of the rat liver showing properties distinct from the major cytosolic enzymes [1,2]. Both membrane-bound enzymes are immunologically related [3,4] and show the same apparent molecular mass [4]. The functional microsomal enzyme has been reported to be present as a trimer consisting of three identical 17.2 kDa polypeptides [5–7]. The activity of the microsomal and the outer mitochondrial membrane enzyme towards 1-chloro-2,4-dinitrobenzene can be stimulated by SH-alkylating reagents such as *N*-ethylmaleimide [3,4,8]. This activation occurs by covalent binding of NEM to a single cysteine residue (Cys-49) in each polypeptide chain of the protein [5,9].

Membrane-associated GSH *S*-transferases exhibit selenium-independent GSH peroxidase activity [10]. A role of the membrane-bound GSH *S*-transferases in the protection of membranes against lipid peroxides has been suggested [10]. This would be complementary to the cytosolic selenium-dependent phospholipid hydroperoxide GSH peroxidase [11].

In an earlier investigation we observed that isolated rat liver plasma membrane fractions possess GSH *S*-transferase activity, which is retained after washing [12]. Here we report that the residual GSH *S*-transferase activity can be stimulated by NEM, similar to the microsomal enzyme, and we demonstrate that the enzyme activity is predominantly localized in the canalicular part of the plasma membrane.

Materials and Methods

Materials. 1-Chloro-2,4-dinitrobenzene and *N*-ethylmaleimide were obtained from Merck (Darmstadt, Germany), iodoacetamide from Schuchardt (Munich, Germany), glutathione from Boehringer-Mannhein (Mannheim, Germany), *t*-butyl hydroperoxide and cumene hydroperoxide from Sigma (Munich, Germany). Protein low molecular weight calibration kit was obtained from Pharmacia LKB (Uppsala, Sweden) and nitrocellulose paper NC45 from Serva (Heidelberg, Germany). Antiserum against rat liver microsomal GSH *S*-transferase was a generous gift from Dr. R. Morgenstern, Department of Toxicology, Karolinska Institute, Stockholm. Western blot alkaline phosphatase system was purchased from Promega (Madison, WI, USA).

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; IAA, iodoacetamide; NEM, *N*-ethylmaleimide.

Preparation of rat liver plasma membranes. Canalicular and sinusoidal plasma membranes were prepared from male Wistar rats (200–300 g body wt.) fed with Altromin standard diet ad libitum, as described by Meier et al. [13]. Both membrane preparations were resuspended in a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ (buffer A). After isolation, the membrane fractions were washed as follows: membranes were diluted 50 times with 100 mM Tris-HCl (pH 7.4) and centrifuged for 40 min at $100\,000 \times g$ in order to remove cytosolic contamination. The resulting pellets were resuspended in 100 mM Tris-HCl (pH 7.4) using a 20 gauge needle and recentrifuged. The pellets were finally resuspended in buffer A using a 26 gauge needle. All operations were performed at 0–4°C. Washed and unwashed membranes were shock frozen with liquid nitrogen and stored at −70°C.

Preparation of subcellular fractions. Total microsomes were isolated as described in Cadenas and Sies [14] and washed as described above for plasma membrane vesicles. Nuclei, whole mitochondria and outer mitochondrial membranes were prepared using published procedures [15–17].

Enzyme activities. GSH *S*-transferase was determined using the method of Habig et al. [18] with CDNB as a substrate. GSH peroxidase was measured with *t*-butylhydroperoxide and cumene hydroperoxide as substrate according to Günzler et al. [19]. In the case of GSH *S*-transferase measurement NEM treatment was performed by incubating 1.0 mg/ml protein with 1 mM NEM for 1 min at room temperature. Samples treated with IAA (1 mM) were incubated for 90 min. The volumes taken from the NEM or IAA incubation mixtures were never more than 0.01 volume of the GSH *S*-transferase assay medium. Alkaline phophatase, γ-glutamyl transpeptidase, Na^+/K^+-ATPase were determined as in Refs. 20–22. NADPH–cytochrome-*c* reductase and succinate–cytochrome-*c* reductase were assayed using the method of Sottocasa et al. [23]. Glucose-6-phosphatase was measured as described by Aronson et al. [24], the release of inorganic phosphate was assayed according to the method of Baykov et al. [25]. The assay of Weissbach et al. [26] was used for the determination of monoamine oxidase. NAD^+ pyrophosphorylase was assayed to estimate the contamination with the nuclear envelope [27]. Lactate dehydrogenase was measured using the procedure described by Bergmeyer et al. [28]. Protein was determined by the method of Bensadoun and Weinstein [29].

Gel electrophoresis and protein transfer. Proteins were separated by SDS-PAGE using a 15% polyacrylamide gel according to Laemmli [30]. Proteins were then

TABLE I

Characterization of washed canalicular and sinusoidal plasma membrane vesicles

The percentage of contamination was calculated by setting the marker enzyme activities of the isolated organelles as 100%. Values are means ± S.E. from three independent experiments.

Marker enzyme	Canalicular		Sinusoidal	
	mU/mg	% contamination	mU/mg	% contamination
Canalicular				
Alkaline phosphatase	209 ± 5		27 ± 4	13
γ-Glutamyltranspeptidase	81 ± 16		17 ± 5	21
Sinusoidal				
Na^+/K^+-ATPase	7 ± 6	4	164 ± 29	
Microsomal				
NADPH-cytochrome-*c* reductase	11 ± 3	5	4 ± 1	2
Glucose 6-phosphatase	31 ± 1	6	3 ± 1	0.6
Mitochondrial [a]				
Inner membrane				
Succinate–cytochrome-*c* reductase	20 ± 4	0.8	266 ± 21	11
Outer membrane				
Monoamine oxidase	7 ± 0.5	4	6 ± 0.8	3
Nuclear				
NAD pyrophosphorylase [b]	2.2 ± 0.5	1.6	2.3 ± 0.5	1.8
Cytosolic				
Lactate dehydrogenase	178 ± 14	3	250 ± 11	5

[a] Calculated assuming that outer and inner mitochondrial membranes represent 4 and 21% total mitochondrial protein, respectively [36].
[b] Enzyme activity in nmol/mg per h.

TABLE II

Effect of NEM and IAA treatment on GSH S-transferase activity in rat liver membrane fractions

NEM treatment was performed as described in Materials and Methods. Values are means ± S.E. from three experiments.

Membrane fraction	GSH *S*-transferase activity (mU/mg protein)					
	unwashed	washed				
		control	NEM treated	NEM treated/ control	IAA treated	IAA treated/ control
Canalicular	337 ± 27	83 ± 8	202 ± 22	2.4	165 ± 10	2.0
Sinusoidal	496 ± 31	50 ± 3	115 ± 9	2.3	83 ± 7	1.7
Microsomal	251 ± 31	71 ± 8	517 ± 21	7.3	266 ± 34	3.7
Outer mitochondrial [a]	–	260 ± 41	1058 ± 129	4.1	613 ± 92	2.4

[a] Isolation procedure includes washing [17].

transferred to nitrocellulose paper at 1 mA/cm^2 for 90 min by the method of Kyhse-Andersen [31]. Proteins bound to nitrocellulose paper were stained with Indian Ink [32]. For immunodetection duplicate nitrocellulose filters were probed with antisera against the microsomal GSH *S*-transferase. The antigen-antibody com-

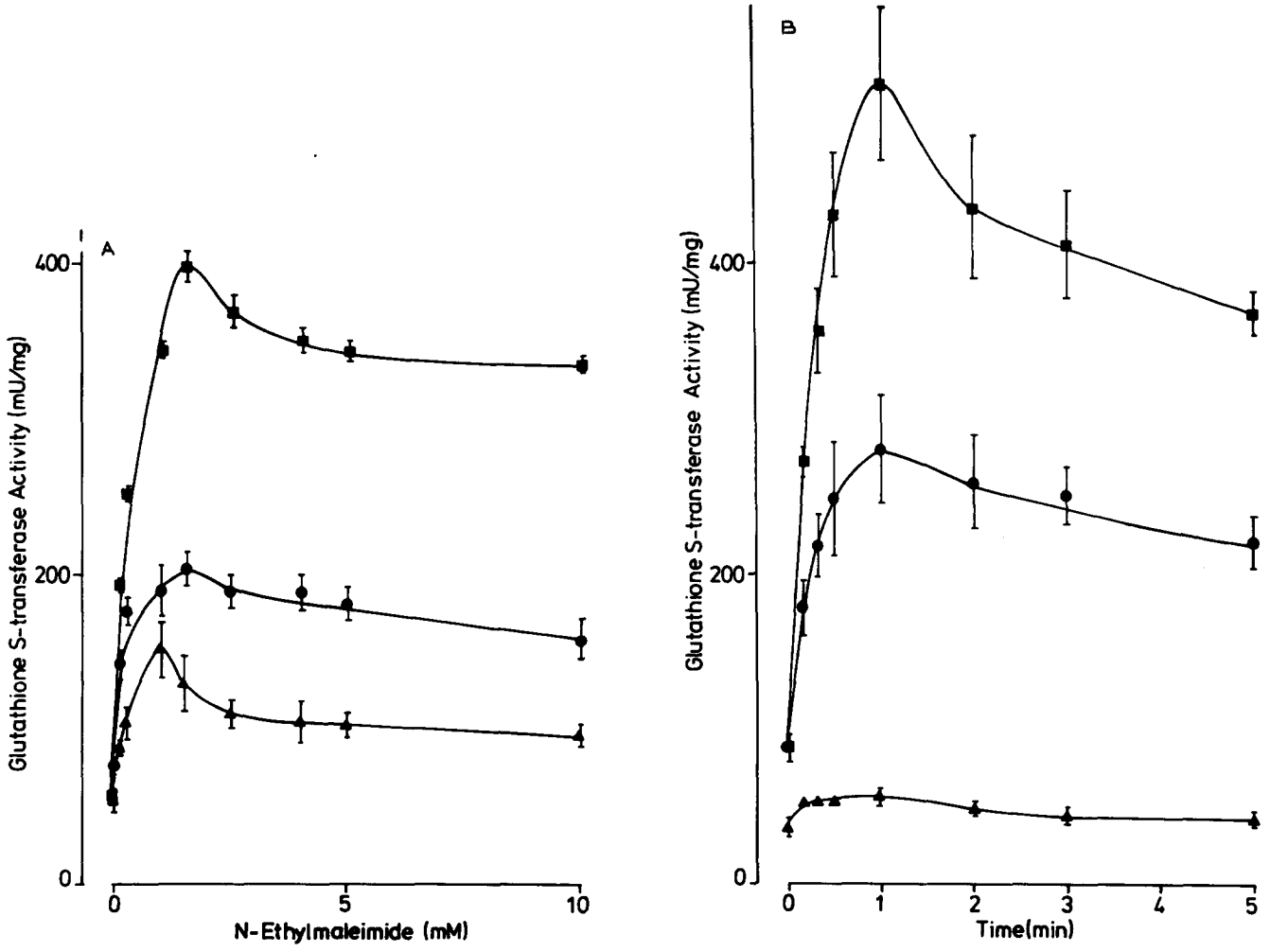

Fig. 1. Concentration (A) and time-dependence (B) of NEM on GSH *S*-transferase activity in rat liver microsomes (■), canalicular (●) and sinusoidal membranes (▲). NEM treatment was performed for 1 min (A) and with 1 mM NEM (B). Data are means ± S.E. from three experiments.

plexes were visualized with the alkaline phosphatase system.

Results

Characterization of plasma membrane preparations

Canalicular plasma membranes are highly enriched in the marker enzymes alkaline phosphatase and γ-glutamyl transpeptidase (see Ref. 13 and Table I). As calculated from the residual Na^+/K^+-ATPase activity, the canalicular membrane preparation contains about 4% of sinusoidal membranes. The content of microsomal or mitochondrial membranes in the canalicular membrane preparation amounts to 6 and 4%, respectively. The contamination by nuclear membranes is even lower (1.6%).

The sinusoidal plasma membrane preparation contains 13–20% membranes of canalicular origin and a relatively high amount of inner mitochondrial membranes (11%). Using the washing procedure as described in Materials and Methods, the cytosolic contamination is diminished to 3% and 5% for the canalicular and sinusoidal membranes, respectively. No further release of cytosolic marker enzymes is observed by additional washing with 0.25 M NaCl.

Glutathione transferase activity in plasma membranes

GSH *S*-transferase activities in the membrane fractions are substantially lowered by repeated washing (see Ref. 33 and Table II). Whereas in sinusoidal membranes the activity is decreased to 10%, the activity is lowered to 25% in the canalicular membrane fraction and to 35% in the microsomal preparation. Lactate dehydrogenase activity decreased in parallel (data not shown). The membrane-associated glutathione *S*-transferase activity is stimulated by the sulfhydryl reagents *N*-ethylmaleimide and iodoacetamide (Table II). Whereas the specific activity is highest in the outer mitochondrial membranes, substantial activity is observed in the canalicular plasma membrane fraction. The activity in the sinusoidal membranes is much lower. Furthermore, according to calculations on the basis of marker enzyme analysis about 60–70% of the activity in the sinusoidal fraction may be ascribed to contaminating membranes. For the canalicular membranes this is maximally 20–30%. Thus, the activity in the plasma membrane fractions retained after washing must be attributed to an intrinsic membrane-bound GSH *S*-transferase, predominantly present in the canalicular domain.

Stimulation by sulfhydryl reagents

To establish the maximum activation of GSH *S*-transferase by *N*-ethylmaleimide, the concentration-dependence and the time-course for activation were examined. As shown in Fig. 1A, microsomes, as well as canalicular and sinusoidal membranes are stimulated maximally between 1.0–1.5 mM NEM. At higher concentrations the stimulation of the GSH *S*-transferase activity is decreased. The activation of the GSH *S*-transferase in the three membrane fractions occurs rapidly within 1 min (Fig. 1B). At longer incubation times the increase of the activity is lowered.

TABLE III

GSH peroxidase activities in different rat liver membrane fractions

NEM incubation was performed with 1.5 mg/ml protein. Values are means ± S.E. (n = 3–5).

Membrane fraction	GSH peroxidase (mU/mg protein)			
	t-butyl hydroperoxide		cumene hydroperoxide	
	control	NEM-treated	control	NEM-treated
Canalicular	19±4	13±4	32±5	26± 3
Sinusoidal	45±6	42±6	56±9	50± 5
Microsomal	14±2	24±5	20±4	35± 7
Outer mitochondrial	12±3	13±2	44±7	69±13

Glutathione peroxidase activity

The enzymes were further characterized by their substrate specifity towards hydroperoxides. The microsomal enzyme exhibits a GSH peroxidase activity which can be stimulated two-fold by NEM both with cumene hydroperoxide [10] and with *t*-butyl hydroperoxide as a substrate (Table III). Furthermore, canalicular and sinusoidal plasma membranes exhibit GSH peroxidase activity with both hydroperoxides. However, in contrast to microsomes the GSH peroxidase activity in the plasma membranes is slightly inhibited by NEM. The mitochondrial activity is activated by NEM only with cumene hydroperoxide as the substrate.

Western blot analysis

Morgenstern et al. [3] and Nishino and Ito [4] demonstrated that the GSH *S*-transferase of the microsomal and the outer mitochondrial membranes are immunochemically related. Therefore, canalicular, sinusoidal and mixed membrane preparations [13] were tested by Western blot analysis with an antibody against the microsomal GSH *S*-transferase (kindly provided by Dr. R. Morgenstern, Stockholm). Fig. 2 illustrates that a 17 kDa protein is labeled in the three plasma membrane fractions (Fig. 2A, lanes 1–3), corresponding to the molecular mass of 17237 Da for the microsomal transferase deduced from sequence analysis of the protein [5].

Fig. 3 gives an estimation of the relative amounts of membrane bound GSH *S*-transferase in the canalicular, sinusoidal and microsomal membranes. A dilution series of 5 to 0.63 μg protein of each fraction was tested with an antiserum against the microsomal GSH

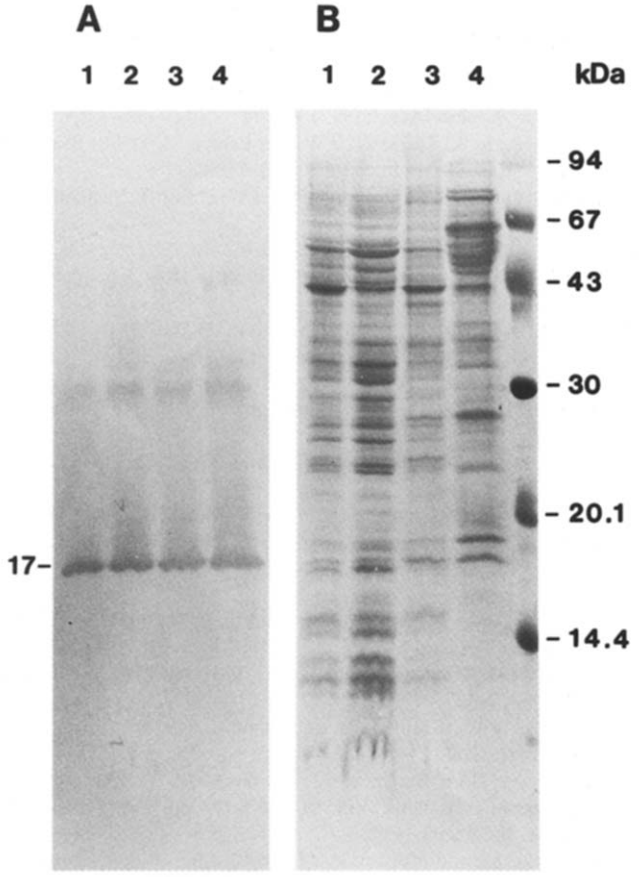

Fig. 2. Western blot (A) and protein stain (B) of mixed membranes (lane 1), sinusoidal (lane 2) and canalicular membranes (lane 3) and microsomes (lane 4) after electrophoretic separation. 150 μg protein per lane was transferred to nitrocellulose paper for immunodetection, duplicate nitrocellulose filter with 200 μg protein per lane was stained with Indian Ink.

S-transferase. Densitometric analysis of the intensity of the antibody reaction in corresponding dilutions of the different plasma membrane fractions indicates that the amount of GSH *S*-transferase in the canalicular membranes is 20 μg/mg protein and in the sinusoidal membranes about 5–10 μg/mg protein. The amount of GSH *S*-transferase in microsomes amounts to 31 μg/mg protein [3].

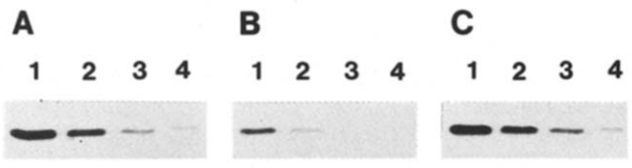

Fig. 3. Quantitation of GSH *S*-transferase in canalicular (A), sinusoidal (B) and microsomal membranes (C) with immunoblot analysis. 5, 2.5, 1.25 and 0.63 μg (lanes 1–4) were probed with antiserum against GSH *S*-transferase. The antibody reaction was visualized with alkaline phosphatase system.

Discussion

Rat liver plasma membranes exhibit substantial membrane-bound glutathione *S*-transferase activity, localized mainly in the canalicular domain (Table II). Since NEM inhibits cytosolic GSH *S*-transferase activity [34], the stimulation of canalicular and sinusoidal GSH *S*-transferase by NEM clearly indicates that the enzyme activity in both membrane preparations is not due to cytosolic contamination. Also, the GSH *S*-transferase activity recently described to be present in rat liver nuclei [35] is inhibited by NEM.

Cytosolic contamination may be partially responsible for the biphasic behavior of the time course and concentration dependence for NEM activation as shown in Fig. 1. While the first phase represents activation of the membrane-bound enzyme, inhibition of cytosolic or other GSH *S*-transferases might occur in the second phase. The presence of GSH *S*-transferases, which are either stimulated or inactivated by NEM, could also offer an explanation for the finding that the extent of activation by NEM is quite different in the different membrane preparations (Table II). The activity of the microsomal enzyme can be increased seven-fold by NEM and four-fold by IAA (see Ref. 8 and Table II). The enzyme activity of the mitochondrial outer membrane was stimulated four- to five-fold by NEM preincubation (see Ref. 3 and Table II). The activities in the isolated plasma membranes can be enhanced by NEM and IAA by a factor of two (Table II). The extent of the stimulation is known to increase upon isolation. For example, purified microsomal and mitochondrial GSH *S*-transferase exhibit higher specific activity and NEM stimulation (10–15-fold) than the corresponding membrane fraction [4,9].

Friedberg et al. [33] estimated the activity in rat liver plasma membranes to be only 10% of the activity present in the endoplasmic reticulum. However, they used a different isolation procedure and did not distinguish between canalicular and sinusoidal parts of the membrane. With highly-purified canalicular plasma membranes (Table I) we observe a specific basal GSH *S*-transferase activity of 83 ± 8 mU/mg protein, higher than that in microsomes (Table II). The activity in the sinusoidal plasma membranes, comprising the major part of the plasma membrane, is much lower. The value of 50 ± 3 mU/mg protein includes substantial contribution (possibly up to 70%) by contaminating membranes.

The isolated microsomal and mitochondrial GSH *S*-transferase were reported to act as a selenium-independent GSH peroxidase, which can be stimulated by NEM with cumene hydroperoxide as a substrate [4,9]. In contrast, GSH peroxidase activity in plasma membranes is slightly inhibited by NEM (Table III). This could indicate that the canalicular GSH *S*-transferase

is different from the microsomal and mitochondrial enzyme and that the GSH peroxidase activity may be attributed to the selenium-dependent phospholipid hydroperoxide GSH peroxidase [11]. However, it is also possible that the stimulation escapes detection because of the low activity in the membrane preparations.

Morgenstern et al. [3] tested the various membranes of hepatocytes for the presence of GSH *S*-transferase with an antibody raised against the microsomal enzyme. The highest level was detected in the outer mitochondrial membranes and a lower level in the microsomes. However, no cross-reaction was observed with plasma membranes. Using the same antibody, but another plasma membrane isolation procedure, we were able to detect a signal at about 17 kDa in canalicular and sinusoidal membrane preparations (see Fig. 2A, lanes 1–3). The reason for this discrepancy is not clear. The presence of significant glutathione *S*-transferase activity in isolated rat liver plasma membranes has been documented [33]. Our canalicular and sinusoidal preparations are contaminated only by 6 and 2%, respectively, with microsomal membranes (Table I), so that the antibody reaction can not be attributed to a microsomal contamination. As illustrated in Fig. 3, the amount of GSH *S*-transferase in the canalicular membranes is about three-fold higher than in the sinusoidal membranes, corresponding well with the activity measurements (Table II).

These data support the conclusion that the immunologically detectable 17 kDa protein is identical with the GSH *S*-transferase activity present in the isolated plasma membranes.

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