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S-(4-Azidophenacyl)[^{35}S]glutathione photoaffinity labeling of rat liver plasma membrane-associated proteins

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A method for the synthesis of the glutathione conjugate *S*-(4-azidophenacyl)[^{35}S]glutathione is described. The compound was used for photoaffinity labeling of proteins present in canalicular membrane vesicles (CMV), sinusoidal membrane vesicles (SMV), mitochondria and microsomes from rat liver Most of the radioactivity introduced by photoaffinity labeling of CMV appeared in the 25–29 kDa range. Further labeled proteins were observed in bands at 37, 105 and about 120 kDa. 79% of the 25–29 kDa associated radioactivity was recovered in the supernatant after extensive revesiculation (washing) of the vesicles, together with the 37 kDa protein CMV and SMV contained glutathione *S*-transferase (GST) activity which in CMV was decreased by 75% by washing. Photolabeling of a mixture of purified basic GST subunits from rat liver resulted in a band pattern at 25–29 kDa similar to that in the membrane preparations. Isoelectric focusing of the CMV indicated the presence of basic soluble GST subunits *S*-Hexylglutathione-Sepharose affinity chromatography showed reversible binding of photolabeled proteins at 25–29 kDa. Difference photoaffinity labeling with GSSG, *S*-hexylglutathione, taurocholate and phenylmethylsulfonyl fluoride decreased the radioactivity bound by GST, but not that introduced into the 105 kDa protein band present in CMV. It is concluded that membrane-associated basic GST isoenzymes are present in standard membrane vesicle preparations. In the cell, the function may be transport of GST-bound compounds across the membrane and protection of the membranes against electrophiles.

Introduction

Based on previous work [1,2], several methods for the isolation of plasma membrane preparations enriched in marker enzymes either from the canalicular (biliary) or the sinusoidal (basolateral) membrane poles of rat hepatocytes have recently been described [3–6] Plasma membrane vesicles were used in transport studies with numerous compounds including hydrophilic substrates such as L-alanine [4,7,8], glutathione [9], GSSG [10], and amphipathic compounds, e g , *S*-(2,4-dinitrophenyl)glutathione [11] or taurocholate [8,12,13] Binding studies with cholate derivatives have also been performed [14]

The technique of photoaffinity labeling has been used for the isolation of a 100 kDa protein from CMV using a radioactive photolabile derivative of taurocholate [15,16] Reconstitution experiments showed that this protein catalyzes taurocholate transport into liposomes [17]

The purpose of this work was to elaborate a method for the synthesis of a radioactive photoaffinity label for the detection of glutathione S-conjugate-binding membrane proteins, possibly involved in the transport of these glutathione species across the plasma membrane. In the present study, *S*-(4-azidophenacyl)[^{35}S]glutathione ([^{35}S]APASG) was used for photoaffinity labeling of proteins present in CMV and SMV from rat hepatocytes APASG in its nonradioactive form [18] has been shown to inactivate purified cytosolic glyoxalase I (EC 4 4 1 5) and II (EC 3 1 2 6) and glutathione *S*-transferase (GST) (EC 2 5 1 18) activity irreversibility during photoaffinity labeling [19,20] The presence of GST activities in membrane preparations was not examined

Abbreviations APASG, *S*-(4-azidophenacyl)glutathione, [^{35}S] APASG, *S*-(4-azidophenacyl)[^{35}S]glutathione, CDNB, 1-chloro-2,4-dinitrobenzene, CMV, canalicular membrane vesicles, GST, glutathione *S*-transferase(s), γ-GT, γ-glutamyl transpeptidase, HexSG, *S*-(*n*-hexyl)glutathione, IEF, isoelectric focusing, IEP, isoelectric point, PMSF, phenylmethylsulfonyl fluoride

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so far An update on current knowledge on GST is given in Refs 21 and 22

Methods and Materials

(1) Synthesis of [^{35}S]APASG

Operations were carried out avoiding exposure to direct light The steps prior to the first TLC were conducted under nitrogen and with nitrogen-saturated solutions [^{35}S]glutathione (125 μCi) was freed from dithioerythritol (DTE) [23] and was diluted with nonradioactive GSH to a specific activity of 40 Ci/mmol, the final volume was 40 μl Derivatisation was started by the subsequent addition of 10 μl 4-azidophenacylbromide (final concentration 4 mM), 50 μl potassium phosphate buffer (pH 7 2)/EDTA (final concentrations 50/1 mM), 5 μl glutathione reductase (120 mU) and 5 μl NADPH (final concn 1 mM) The reaction was allowed to proceed at room temperature and was stopped after 20 min by applying the reaction mixture to a Polygram Sil G TLC plate that was developed in 1-propanol/water/acetic acid (12 5 1, v/v) (solvent system A) The area on the TLC plate containing the radioactivity corresponding to unlabeled APASG (R_f = 0 60) was scraped off and extracted six times with 800 μl water (50 °C) The extract was filtered through a Millex-GS 0 22 μm filter and lyophilised to decrease the volume to about 50 μl A second TLC was carried out using 1-butanol/water/acetic acid (7 2 1, v/v) (solvent system B) for further purification The product that ran parallel to unlabeled APASG (R_f = 0 24) was extracted and filtered as described The radiochemical purity of the product was checked by TLC in solvent systems A and B and system C (1-propanol/water (7 3, v/v), R_f = 0 56) The yield was about 30% and the radiochemical purity more than 95% as determined with an Isomess TLC Analyzer (Isomess GmbH, Straubenhardt, F R G) As the product was prone to decomposition with a rate of approx 5% per day at −70 °C, aliquots of 2–3 μCi were lyophilized in quartz tubes and used immediately for photoaffinity labeling

(2) Preparation of rat liver plasma membrane vesicles and cell compartments

Canalicular and sinusoidal rat liver plasma membrane vesicles (CMV and SMV) were isolated from livers from male Wistar rats (260–300 g body wt) fed an Altromin standard diet ad libitum Six livers were homogenized and membrane separation was performed by a combination of rate zonal flotation and high-speed centrifugation through a discontinous sucrose gradient essentially as described in Refs 5 and 13 *Normal* CMV were taken up in buffer A (313 mM sucrose, 6 25 mM magnesium gluconate, 0 25 mM calcium gluconate, 12 5 mM Hepes-Tris (pH 7 4)) after the final centrifugation *Washed* CMV were obtained by dilution of the CMV with buffer A (1 3 (v/v)) and vesiculation through a 26 gauge syringe needle for 10 times before the final centrifugation

The pellets thus obtained were resuspended in buffer A and were used freshly for photoaffinity labeling with the exception of the samples used for Fig. 2 where vesicles were shock frozen with liquid nitrogen and stored at −70 °C until used Marker enzyme activities and relative enrichment factors with regard to liver homogenate are documented in Table I Mitochondria were isolated by the method of Klingenberg and Slenczka [24], microsomes as described in Ref 25 Both were stored frozen in buffer A at −70 °C until used

(3) Photoaffinity labeling with [^{35}S]APASG, electrophoresis and autoradiography

Vesicles or subcellular preparations were used fresh or after rapid thawing at 37 °C

TABLE I

Marker enzyme activities of rat hepatic canalicular and sinusoidal membrane vesicles

Data are means ± S D n d , not detectable

Sample	Enzyme	Specific activity (mU/mg protein)	Enrichment (-fold increase relative to liver homogenate)	Number of vesicle preparations
CMV				
Normal	Alkaline phosphatase	54 9 ± 15 2	37 9 ± 6 9	6
	Na^+/K^+-ATPase	n d	0	6
	Mg^{2+}-ATPase	1538 ± 300	57 3 ± 10 2	6
	γ Glutamyl transpeptidase	38 5 ± 10 0	38 1 ± 13 4	6
Washed	Alkaline phosphatase	46 0 ± 6 2	29 5 ± 6 1	3
	Na^+/K^+-ATPase	n d	0	3
	Mg^{2+}-ATPase	1180 ± 271	27 6 ± 9 2	3
	γ-Glutamyl transpeptidase	24 4 ± 4 5	23 4 ± 4 5	3
SMV	Alkaline phosphatase	10 4 ± 2 1	7 8 ± 0 8	3
	Na^+/K^+-ATPase	184 0 ± 21 0	10 5 ± 3 8	3
	Mg^{2+}-ATPase	284 0 ± 29 4	14 1 ± 2 1	3
	γ-Glutamyl transpeptidase	7 4 ± 3 8	7 3 ± 3 3	3

The membranes were vesiculated and preincubated at 37°C for 30 min with the γ-GT inhibitor AT-125 (100 μM) in buffer B to avoid enzymatic hydrolysis of the photolabel. Buffer B is 250 mM sucrose, 100 mM potassium gluconate, 5 mM magnesium gluconate, 0.2 mM calcium gluconate, 10 mM Hepes-Tris (pH 7.4). 40 μl of the membrane suspension containing 120–400 μg of protein on ice were added to quartz vials containing [^{35}S]APASG (2–3 μCi/vial) taken up in 160 μl buffer B. Effectors used for difference labeling were added at this stage, solutions being neutralized with concentrated Tris where necessary. The final volume was 200 μl giving concentrations of the photolabel from about 270 to 400 nM. After 2 min of equilibration at room temperature the samples were irradiated for 2 min in a Rayonet RPR-100 Photochemical Chamber Reactor (The Southern New England Ultraviolet Co. Hamden, CT) equipped with 16 symmetrically arranged RPR-3000 Å lamps at ambient temperature without stirring. Aliquots of 5 μl were submitted to TLC analysis either in solvent system A or B to check the state of the photolabel immediately before and after photolysis. Proteins were obtained using the delipidisation procedure of Wessel and Flugge [26] employing methanol and chloroform. In the cases where no organic solvents were used (Fig. 1), the photolabeled membrane suspension was centrifuged for 30 min at 47000 × *g* in a Beckman airfuge (Beckman Instruments GmbH, Munich, F.R.G.). Protein pellets were redissolved in buffer C (125 mM Tris-HCl (pH 6.8), 6.5% SDS, 8.5% glycerol, 5% mercaptoethanol, 0.02% Bromophenol blue) and subjected to electrophoresis with 10% polyacrylamide gels [27]. Gels were stained with Coomassie blue R-250, photographed and dried. The distribution of radioactivity on the dried gels was measured by autoradiography exposing films at −70°C for 2 to 6 days or with a TLC analyzer.

(4) Miscellaneous procedures

Enzyme activities. Marker enzymes (alkaline phosphatase (EC 3.1.3.1), Na^+/K^+ ATPase (EC 3.6.1.3), Mg^{2+}-ATPase and γ-glutamyl transpeptidase (EC 2.3.2.13)) were determined by the methods in Refs. 28–30 (see Table I).

GST activity was assayed as in Ref. 31 by using 1-chloro-2,4-dinitrobenzene (CDNB). Protein was determined as described in Ref. 32.

Treatment of GST subunits. Basic GST isoenzyme subunits 1, 2, 3 and 4 (for nomenclature see Ref. 22) from rat liver were purified according to Refs. 31, 33, 34 and stored frozen at −70°C. After rapid thawing at 37°C, the subunits were mixed to yield a specific activity of 27.2 U/mg. 10 μg of protein were used for photolabeling after dialysis against buffer B and treatment with AT-125 to create uniform conditions.

Affinity chromatography of labeled CMV proteins. Photolabeled CMV were solubilized by 30 min incubation at 37°C with 1% Triton X-100 and 2 mM dithioerythritol (DTE) in buffer B (final volume 800 μl) and applied to a 2 ml *S*-hexylglutathione-Sepharose column equilibrated with 10 mM Tris-HCl (pH 7.4), 2 mM DTE, 1% Triton X-100. Unbound proteins were washed from the column (flow-through fraction) with 5 ml of the same buffer. Unspecifically adsorbed proteins were eluted with 5 ml buffer containing 300 mM NaCl. Finally, a third fraction was obtained with 5 ml buffer containing 300 mM NaCl and 5 mM HexSG. Proteins of each fraction were pelleted as in Ref. 26 and subjected to SDS electrophoresis as described above.

Preparative IEF. Preparative isoelectric focusing on Sephadex G-150 was done with 1 mg of CMV protein as described in Ref. 35, except that 2% Triton X-100 was added. CMV protein was solubilized as described in the previous paragraph. The solution was applied to the preparative IEF plate consisting of Sephadex G-150 containing 2% LKB Ampholyte (pH 5.0–8.0) and 2% Triton X-100, layered on the hydrophobic side of Gel Bond PAG film. The plate was focused with buffer strips containing pH 10 and pH 3 electrode buffers for 60 min at 7°C with limiting values of 1500 V, 44 mA, 18 W, for a 2 mm thick, 20.3 cm long and 16 cm wide gel. The resulting flat gel was dissected into 10 fractions which were collected and diluted with 1 ml of water. The pH was measured for coarse IEP determination. After centrifugation the protein in the supernatant was precipitated [26] and the protein fractions were subjected to SDS electrophoresis.

(5) Chemicals

[^{35}S]Glutathione and [^{3}H]taurocholate were from NEN (Dreieich, F.R.G.). Gel Bond PAG Film was from LKB Producter AB (Bromma, Sweden). 4-Azidophenacylbromide was from Fluka (Buchs, Switzerland). Acivicin (AT-125) was a gift from The Upjohn Co. (Kalamazoo, MI). Autoradiography films (Kodak X-OMAT AR) were from Eastman Kodak Co. (Rochester, NY). The Electrophoresis Kit for Molecular Weight Determination of Low Molecular Weight Proteins and Ampholine pH 5–8 were from Pharmacia AB (Uppsala, Sweden). The pH 10 and 3 electrode buffers were from Serva (Heidelberg, F.R.G.).

S-Hexylglutathione-Sepharose 6B was prepared as described in Ref. 33.

For TLC we used Polygram Sil G without gypsum, 0.25 mm (Macherey & Nagel Co, Duren, F.R.G.). To remove silica gel particles from photolabel preparations we used Millex-GS 0.22 μm filters (Millipore S.A., Molheim, France). Filters for taurocholate export were 0.45 μm cellulose nitrate filters (Type SM 11306, Sartorius GmbH, Gottingen, F.R.G.).

All other chemicals were from Merck (Darmstadt, F R G), Boehringer (Mannheim, F R G), and Serva (Heidelberg, F R G)

Results

Synthesis and characterization of the photolabel

For the synthesis of the radioactive glutathione-derived photolabel, *S*-(4-azidophenacyl)[^{35}S]glutathione, an excess of azidophenacylbromide over [^{35}S]glutathione and the presence of GSSG reductase and NADPH improved the yield considerably Characterization of the product by TLC demonstrated a radiochemical purity larger than 95% The product decomposed with a rate of approx 5% per day, probably due to release of nitrogen. and therefore was used immediately for photoaffinity labeling experiments The advantages of the ^{35}S-labeled conjugate as compared to ^{3}H-labeling are the high specific activity and the more intensive β-irradiation To avoid hydrolysis of the intact photoaffinity label by γ-GT, we preincubated all membrane preparations in the presence of the γ-GT inhibitor acivicin (AT 125) [11] After 2 min incubation of the photolabel with the pretreated CMV more than 90% of the radioactivity could be identified as APASG by TLC using solvent system B Without AT-125 only 65% of the radioactivity migrated with unlabeled APASG

Dependence of labeling pattern on procedure of vesicle preparation and processing

Using the radioactive photoreactive glutathione conjugate *S*-(4-azidophenacyl)[^{35}S]glutathione ([^{35}S]-APASG) for photoaffinity labeling we found differences in the distribution pattern of radioactivity on the autoradiograms from freshly prepared unfrozen *normal* and *washed* CMV (Fig 1) The Coomassie stain (Fig 1A) shows that *normal* CMV (lanes 1, 2) contained a dominant protein band in the 37 kDa range when protein obtained by precipitation with organic solvents (methanol, chloroform [26]) was applied The amount of this 37 kDa protein was substantially decreased after centrifugation without organic solvents (lanes 3, 4), correspondingly, the supernatant contained the 37 kDa protein (lanes 5, 6) In addition, some material in the 25–29 kDa range was dissociable as well In line with this, *washed* vesicles (see Methods) contained low amounts of these proteins (lanes 7, 8), which could be further decreased by pelleting without organic solvents (lanes 9, 10) Fig 1B shows the corresponding autoradiogram By pelleting the CMV protein with organic solvents most of the radioactivity was recovered in the 25–29 kDa range (lanes 1, 2) The amount of the radioactivity of *washed* CMV in this M_r range (lanes 7, 8) was 21% of that of *normal* CMV, 79% appearing in the supernatant (lanes 5, 6) (deviation in two experiments < 3%) In both *normal* and *washed* CMV this radioactivity was further diminished by pelleting without organic solvents (lanes 3, 4 and 9, 10, respectively) The radioactivity seen at the position of the 37 kDa protein in *normal* CMV is also decreased by washing On the other hand, the protein bands at 105 and 120 kDa were not dissociated from the vesicles by any of these procedures, indicating that these proteins are very tightly bound membrane proteins

As examined on TLC, photolysis resulted in the diminution of the content of [^{35}S]APASG from 92 ± 1%

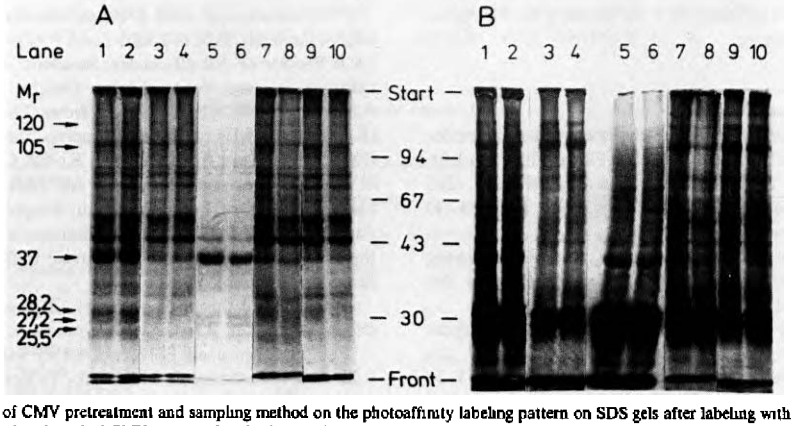
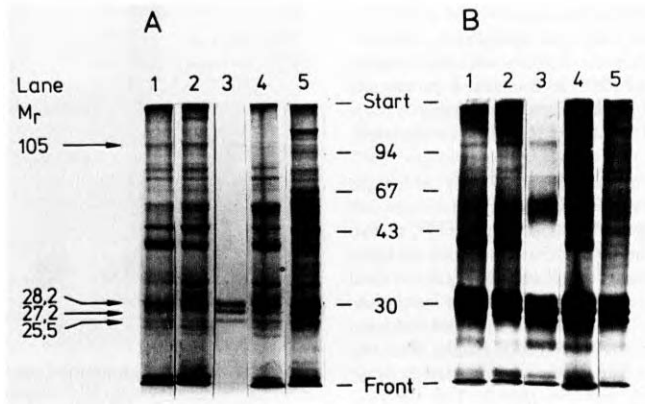


Fig. 1 Influence of CMV pretreatment and sampling method on the photoaffinity labeling pattern on SDS gels after labeling with [^{35}S]APASG (3 μCi/lane) *Normal* and *washed* CMV were used with 180 μg of protein per lane (A) Coomassie stain. (B) Corresponding autoradiogram Lanes 1, 2 *normal* CMV precipitated according to Ref 26, lanes 3, 4 *normal* CMV pelleted by centrifugation, lanes 5, 6 supernatant of *normal* CMV pelleted according to Ref 26 Lanes 7, 8 *washed* CMV precipitated according to Ref 26, lanes 9, 10 *washed* CMV pelleted by centrifugation M_r is given ($\times 10^{-3}$)



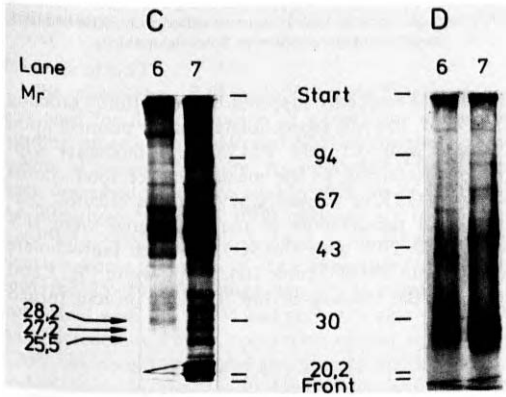


Fig 2 Comparison of the photoaffinity labeling pattern of *normal* CMV (lane 1), *washed* CMV (lane 2), purified GST (lane 3) SMV (lane 4), liver homogenate (lane 5) mitochondria (lane 6) and microsomes (lane 7) after photoaffinity labeling with 2 8 μCi per lane of [^{35}S]APASG except for liver homogenate (1 5 μCi) and mitochondria or microsomes (2 6 μCi) 200 μg of frozen and thawed protein were contained within each sample with the exception of GST (10 μg) and microsomes or mitochondria (150 μg) (A, C) Coomassie stain (B D) autoradiogram

immediately before irradiation to 8 3 ± 0 6% afterwards, 41 ± 2% of the radioactivity now appearing at the origin of the TLC plate and 51 ± 2% at $R_f = 0\,25$ ($n = 8$) [^{35}S]APASG alone ($R_f = 0\,60$) was 96 3% pure (data from two experiments)

TABLE II

GST-activity in Normal and Washed CMV and SMV

Vesicles	GST activity (mU/mg ±S D)	Total protein (mg±S D)	Number of vesicle preparations
Liver homogenate	700±99	12700 ±1100	14
CMV			
Normal	304±76	92±28	14
Washed	78±15	87±27	9
SMV	339±55	319±36	13

Presence of GST in different cell fractions

GST activity

On the autoradiogram, the strongest labeling was found for CMV proteins with $M_r = 28\,2$, 27 2, and 25 5 kDa (Fig 1) This is the range typical for the soluble GST isoenzyme subunits GST activity was present in *normal* CMV and SMV with a specific activity comparable to that of total liver homogenate (Table II) After washing, the amount of activity in CMV was lowered to about 25% This decrease correlated with the decrease of radioactivity found in the 25–29 kDa range seen by washing of CMV (Fig. 1), suggesting that the labeling was due to GST subunits

Preparative IEF

Unlabeled 25–29 kDa proteins in *normal* CMV were also found by preparative IEF of Triton X-100-solubi-

lized CMV proteins on a Sephadex G-150 flat bed gel SDS electrophoresis of the pH 10 fraction produced three bands with molecular mass 25 5, 27 2 and 28 2 kDa (data not shown) This is in accordance with the presence of basic isoenzymes of GST Also the 37 kDa protein was found in the pH = 10 fraction A protein of 165 kDa was present in the most acidic fraction (pH < 5) together with most of the other membrane associated proteins

Comparison of different cell fractions

That binding of photoaffinity label to GST is responsible for most of the radioactivity in the 25–29 kDa range was further evidenced by photolabeling a mixture of the GST subunits 1, 2, 3, and 4 On the lane with purified GST (Fig 2B, lane 3), 78% of the total radioactivity corresponded to the three GST bands The remainder was bound to contaminations not detected by Coomassie blue staining (Fig 2A, lane 3) This demonstrates the high degree of sensitivity of the method for detection of glutathione conjugate binding proteins The GST subunits revealed bands with apparent molecular mass of 25 5 27 2 and 28 2 kDa It can be seen from Fig 2 that SMV, liver homogenate, microsomes and to a weaker extent, mitochondria all show labeling in the 25–29 kDa range with similar band patterns The 37 kDa protein band is present mainly in *normal* CMV and SMV The protein at 105 kDa is present only in CMV

Affinity chromatography of photolabeled CMV proteins

GST subunits are bound to *S*-hexylglutathione-Sepharose affinity columns [33] The labeled proteins in the 25–29 kDa range from *normal* CMV also show this behaviour (Fig 3) 37% of the radioactivity in the GST M_r range was eluted by HexSG in the final elution step 54% of the radioactivity in this range was eluted with the flow-through fraction, 9% with NaCl The labeled 105 kDa protein and the 120 kDa protein also appeared in the flow-through fraction

Difference labeling of CMV

To search for specific effects on the protein labeling, difference photoaffinity labeling of *washed,* freshly prepared CMV with 5 mM GSSG, 5 mM HexSG and 150 μM taurocholate was carried out (Fig 4) Also the serine proteinase inhibitor PMSF (interfering with the activity of the ≈ 100 kDa canalicular protein dipeptidyl peptidase IV) was used 5 mM GSSG decreased the binding to all proteins TLC separation of the photoproducts in the presence of GSSG showed a new peak with $R_f = 0\,11$, whereas the peak at $R_f = 0\,25$ was no longer visible This might indicate some interaction of the photolabel with GSSG or some other side reaction diminishing the yield of the photoreaction A normal TLC pattern was observed with all other effectors 5

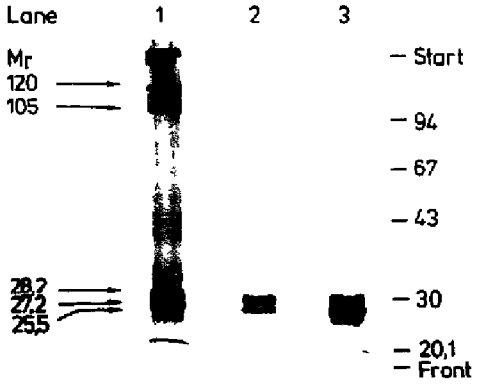


Fig 3 Fractionation of photolabeled proteins from *normal* CMV on *S*-hexylglutathione-Sepharose 6B Autoradiogram of the SDS gel after application of protein obtained by separation of 400 μg of labeled protein after irradiation in the presence of 3 μCi [^{35}S]APASG Lane 1 flow-through fraction, lane 2 proteins eluted with 300 mM NaCl, lane 3 proteins eluted with *S*-hexylglutathione

mM HexSG markedly suppressed photoaffinity labeling of the GST 150 μM taurocholate and the preincubation of CMV with 0 5 mM PMSF gave moderate suppression Referring to the mean value of total counts found for GST in *washed* CMV without effector, percentages of radioactivity in the GST range were 18% with GSSG, 20% with HexSG, 68% with taurocholate and 65% with PMSF (Table III) As shown in Fig 1 and Table III, the labeling of the 105 kDa protein turned

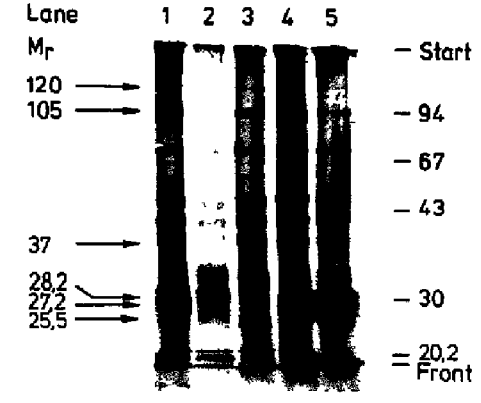


Fig 4 Difference labeling of proteins from *washed* CMV Influence of 5 mM GSSG (lane 2), 5 mM HexSG (lane 3), 150 μM taurocholate (lane 4) and preincubation of CMV with 0 5 mM PMSF at 37 °C for 30 min (lane 5) on photoaffinity labeling of 150 μg protein in the presence of 2 6 μCi of [^{35}S]APASG compared to control without inhibitor (lane 1)

TABLE III

Radioactivity of plasma membrane proteins after difference labeling

Data of two experiments

Sample	Inhibitor	Radioactivity (cpm) at	
		25–29 kDa	105 kDa
Washed CMV	None	1718	61
		1987	64
	GSSG, 5 mM	327	52
		332	52
	HexSG, 5 mM	299	42
		425	45
	Taurocholate	1213	64
		1325	67
	PMSF 0 5mM	1346	50
		1078	48

out to be too weak to allow quantitative conclusions on the effects of the different inhibitors

Discussion

Presence of GST

The photolabel *S*-(4-azidophenacyl)[^{35}S]glutathione was used for the recognition of glutathione conjugate-binding proteins in CMV and SMV from rat liver by means of autoradiography As unlabeled APASG has been described as an irreversible inhibitor of GST [19], we investigated whether these enzymes are responsible for [^{35}S]APASG binding Indeed, several lines of evidence indicate that most of the radioactivity introduced into the 25–29 kDa range can be ascribed to GST copurified with the CMV and probably also with SMV and microsomes Firstly, comparing *normal* with *washed* CMV, we found a parallel decrease of the amount of radioactivity in the 25–29 kDa range and of GST activity (Fig 1, Table II) Secondly, purified GST subunits after photoaffinity labeling showed a similar distribution of radioactivity as the bands at 25–29 kDa in CMV, SMV, liver homogenate,microsomes, and, with less bound radioactivity, mitochondria (Fig 2) Thirdly, isoelectric focusing of unlabeled, Triton X-100 solubilized CMV protein and SDS electrophoresis of the proteins with isoelectric points around pH = 10 gave three protein bands in the 25–29 kDa range, consistent with the properties of the basic forms of purified soluble GST [22] It is of interest that no binding of the label to the membrane integrated microsomal GST with 17 kDa (see chapter 5 in Ref 22) was found here (Fig. 2). Fourthly, fractionation of photolabeled CMV protein on *S*-hexylglutathionyl-Sepharose resulted in binding of a large part of the labeled 25–29 kDa proteins by the column matrix (Fig 3). The binding was reversed by addition of free HexSG Binding on the *S*-hexylglutathionyl-Sepharose matrix is a characteristic of GST and is used for the ir purification [33] It seems unexpected that labeled GST bind to the gel if one assumes that the binding center for HexSG in GST is occupied by the covalently bound photoaffinity label A possible explanation is given by the fact that GST exist as dimers From the specific activity of the photolabel, the amount of protein in the 25–29 kDa range and the amount of radioactivity in this range we estimated that about 1% of the GST subunits is radioactively labeled Thus, the probability that both subunits are labeled is negligible That in our experiment nevertheless most of the protein did not bind to the affinity matrix may be due (a) to the presence of Triton X-100 which might interfere with the quarternary structure of the GST and their binding capacity, (b) to overloading of the column, or (c) to interference due to the other components present in CMV The band pattern of the proteins at 25–29 kDa not bound by the affinity matrix was entirely similar to that of the HexSG-eluted bands (Fig 3)

Regarding the nature of the association of GST with the vesicles both inclusion and sticking to the membranes may contribute In erythrocytes, a Ca^{2+}-dependent binding of GST to the plasma membrane has been reported [36] It is of interest to speculate on the fact that GST might be membrane-associated physiologically, providing a first line of defense against incoming electrophiles and products of membrane lipid peroxidation, e g., 4-hydroxynonenal [37] GST associated to membranes also may bind amphiphilic and hydrophobic substrates at the inner plasma membrane surface prior to transcellular transport

The high affinity photolabeling of GST found with [^{35}S]APASG makes this substance a candidate for the screening of the active or binding center of GST With sulfobromophthalein, a binding-site peptide from GST has been described recently [38]

The presence of GST in vesicle preparations is important for the interpretation of results of transport and binding studies obtained with hydrophobic, amphipathic and GSH-derived substances since GST may contribute a binding component For instance, the difference in the binding properties of cholate and chenodeoxycholate observed with canalicular and sinusoidal membrane preparations [14] is also found for GST and bile acid binders described in Ref 39 For taurocholate transport studies at low concentrations (between 1 and 10 μM [5,8,13]) as compared to reported dissociation constants of 100 μM [39], the contribution of GST-bound taurocholate to the vesicle associated taurocholate during transport may be negligible Indeed we observed that decreasing of the vesicle-associated GST by washing of CMV had no effect on the taurocholate export rate out of vesicles preloaded with 10 μM taurocholate (not shown). On the other hand, transport studies with the CDNB conjugate of GSH, GSH or GSSG with K_d values in the micromolar range for GST

3-3 [40] or with other amphipathic compounds might be complicated by binding to GST For such studies it seems mandatory to determine GST activity in membrane vesicles preparations which has not been done previously, see Refs 1–6

Other proteins

A prominent band with low specific activity after irradiation appeared at 37 kDa The nature of the protein(s) is not known From the finding that it is easily removed from the membrane (Fig 1) it can be concluded that it does not represent an integral membrane protein

The labeling of a non-dissociable 105 kDa CMV protein by [^{35}S]APASG is of interest since a protein of similar molecular mass has been described as a canalicular taurocholate transport protein [16,17] Furthermore, inhibition of taurocholate transport by GSH conjugates has been described [13] Thus the glutathione conjugate photolabeling found in our studies could represent binding to the membrane protein involved in taurocholate transport Proteins of about 100 kDa have also been described as dipeptidylpeptidase IV (EC 3 4 14 5) [41] and as a cell-cell adhesion protein [42] which might bind to collagen One or more proteins might be involved here The substrates for these proteins and also APASG contain hydrophobic moieties and peptide bonds Thus, APASG has the potential to bind to these proteins

Difference labeling

HexSG in a large excess compared to the concentration inhibiting GST activity in Ref 43 suppressed binding of the photolabel to the GST almost completely (Fig 4, Table III) Labeling of the 105 kDa protein was not suppressed significantly as compared with the control Taurocholate showed only moderate inhibition in the GST range and did not diminish labeling at the 105 kDa protein A lack of suppression of labeling of the 105 kDa protein by HexSG or taurocholate may be explained by a compensatory effect due to the higher availability of label not bound by the GST PMSF, an inhibitor of dipeptidylpeptidase IV [44], showed no effect As shown in Results, the effect of GSSG may be due to an interaction between GSSG and the photolabeling reagent

Taken together, the data show specific effects of HexSG and taurocholate on the binding of the photolabel [^{35}S]APASG to GST The label proved to be a sensitive probe for these enzymes and also other binding proteins as demonstrated by the labeling of protein bands (Fig 2) not visible on the Coomassie stain Specific labeling of the 105 kDa protein cannot be deduced from our data. The role of this protein (and also of a further labeled band at about 120 kDa) and conclusions concerning a possible role as a glutathione conjugate transport-involved protein require further experiments

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