The amount and nature of glutathione transferases in rat liver microsomes determined by immunochemical methods

Ralf Morgenstern, Claes Guthenberg, Bengt Mannervik and Joseph W. DePierre

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

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The amount and nature of glutathione transferases in rat liver microsomes were determined using immunological techniques. It was shown that cytosolic glutathione transferase subunits A plus C, and B plus L were present at levels of 2.4 ± 0.6 and 1.5 ± 0.1 μg/mg microsomal protein, respectively. These levels are 10-times higher than those for non-specific binding of cytosolic components judging from the distribution of lactate dehydrogenase, a cytosolic marker. The possibility that a portion of these glutathione transferases is functionally localized on the endoplasmic reticulum is discussed. A previously described microsomal glutathione transferase which is distinct from the cytosolic enzymes is present in an amount of 31 ± 6 μg/mg microsomal protein.

Glutathione transferase *Microsomal* *Rat liver* *Drug metabolism*

1. INTRODUCTION

The glutathione transferases are a family of enzymes with broad and overlapping substrate specificities (review [1]). These enzymes are important in the detoxication of numerous potentially toxic, carcinogenic and mutagenic substances [2] and can also function as binding proteins [3].

To date at least 7 different proteins in rat liver cytosol with glutathione transferase activity have been described [4,5]. It was recently found that the major part of the activity is due to 6 dimeric proteins formed by binary combinations of 4 different subunits, named A, B, C and L [5,6]. The glutathione transferases previously known as A, AA, B and C are now referred to as transferases A_2, B_2, BL and AC, on the basis of their respective subunit compositions. Accordingly, antibodies raised against transferase 'B' (now BL) react with both subunits B and L, and antibodies towards transferase 'C' (now AC) react with subunits A and C [6]. Antibodies towards the heterodimers AC and BL recognize the corresponding homodimers with the same titer, with the exception of L_2, which is underestimated by 34 ± 10% (n = 5, SD) by the immunoquantitative methods used. By use of such antibodies it was previously found that about 5% of the protein in the cytosol fraction of rat liver is accounted for by subunits A, B, C, and L of the glutathione transferases [7]. The purification of soluble glutathione transferases from mitochondria has been described [8], but the relationship of these enzymes to the cytosolic transferases is not yet clear.

Microsomes demonstrate glutathione transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) at a level which is about 10% of that observed in the cytosol. In [9] it was concluded on the basis of immunological and isoelectric focusing experiments that the microsomal glutathione transferases were identical or at least very similar to their cytosolic counterparts, but might have a stable localization on the endoplasmic reticulum since they could not be removed by various washing procedures (see [10]).

In [11], we discovered a new microsomal glutathione transferase activity which can be stimulated several-fold with thiol reagents under conditions where the cytosolic activities are not affected [11]. We also demonstrated that this activity

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is localized on the endoplasmic reticulum and is not affected by xenobiotics which induce the cytosolic glutathione transferase [10].

We purified this new microsomal glutathione transferase in both *N*-ethylmaleimide-activated [12] and unactivated forms [13]. The purification factor of 36 indicates that this protein constitutes as much as 3% of the total protein of the endoplasmic reticulum. This microsomal transferase differs from the cytosolic enzymes in terms of immunochemical properties, M_r, amino acid composition, substrate specificity, and dependence on detergent for activity [12,13].

The fact that both a distinct microsomal glutathione transferase and glutathione transferases very similar or identical to the cytosolic enzymes are recovered in rat liver microsomes raises important questions as to which of the cytosolic enzymes are present, how much of the different transferases are there, and whether the presence of the cytosolic transferases can be explained simply by contamination. Here, we use immunochemical methods to quantitate the amounts of the microsomal glutathione transferase and the major cytosolic glutathione transferases present in microsomes.

2. MATERIALS AND METHODS

Liver microsomes from male Sprague-Dawley rats (180–200 g) were prepared as described previously, except that the microsomes were washed twice with 0.15 Tris–HCl (pH 8) to remove cytosolic contamination [10]. Microsomal glutathione transferase [12] and cytosolic transferases AC ('C') and BL ('B') were purified as in [5,7,14] and antisera raised in rabbits by conventional methods. Glutathione transferase activity with 1-chloro-2,4-dinitrobenzene was measured as in [4]. Protein was measured as in [15], sometimes as modified in [16], with bovine serum albumin as the standard.

Radial immunodiffusion was performed on 8 × 8 cm glass plates on which were cast 8 ml of 1% agar containing 0.125 and 0.2 ml of antisera against glutathione transferase AC and BL, respectively; 1% Triton X-100 and 50 mM Tris–glycine (pH 8.6) (transferase AC) or 50 mM potassium phosphate buffer (pH 7) (transferase BL) were also included. Purified enzymes were used as standards. Wells of 5 mm diameter were punched and 20 μl of different dilutions of standard proteins or microsomes (solubilized with 3 mg Triton X-100/mg protein) was added. The plates were left to stand at room temperature in a humid atmosphere for 48–72 h and after washing in several changes of 1% Triton X-100, 0.9% NaCl and 50 mM potassium phosphate (pH 7) the gels were stained with Coomassie brilliant blue as in [17].

Rocket immunoelectrophoresis for quantitation of the subunits of glutathione transferase BL was performed on 8 × 8 cm glass plates on which were cast 8 ml 1% agar gel containing 0.25 ml of antisera 23 mM barbiturate buffer (pH 8.6), 1% Triton X-100; 5 μl of different dilutions of pure proteins or microsomes were added to the wells as described above. The gel was run overnight at 4°C with 4 V/cm. Gels were washed and stained as above.

The amount of microsomal glutathione transferase was determined by immunoblotting [18] after sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis (15%) as in [17]. The amount of protein on the blot was quantitated by the use of radioiodinated protein A.

Fig.1 shows standard curves for typical experiments with all 3 techniques.

All chemicals used were from common commercial sources and of the highest purity available. Radioiodinated protein A was a generous gift from Dr A. Wielburski of this department.

3. RESULTS AND DISCUSSION

In this study 3 different immunochemical methods for enzyme quantitation were used. The glutathione transferases of the A–C group [5] have isoelectric points close to immunoglobulins and are measured by radial immunodiffusion. Glutathione transferases of the B-L group have higher isoelectric points and hence can be estimated both by rocket immunoelectrophoresis and radial immunodiffusion. Antisera against the microsomal glutathione transferase also recognize a few microsomal peptides of higher M_r and therefore immunoblotting after separation on SDS–PAGE is a specific way to measure the content of this enzyme.

The results summarized in table 1 show that the microsomal glutathione transferase accounts for 3.1% of the total protein in rat liver microsomes

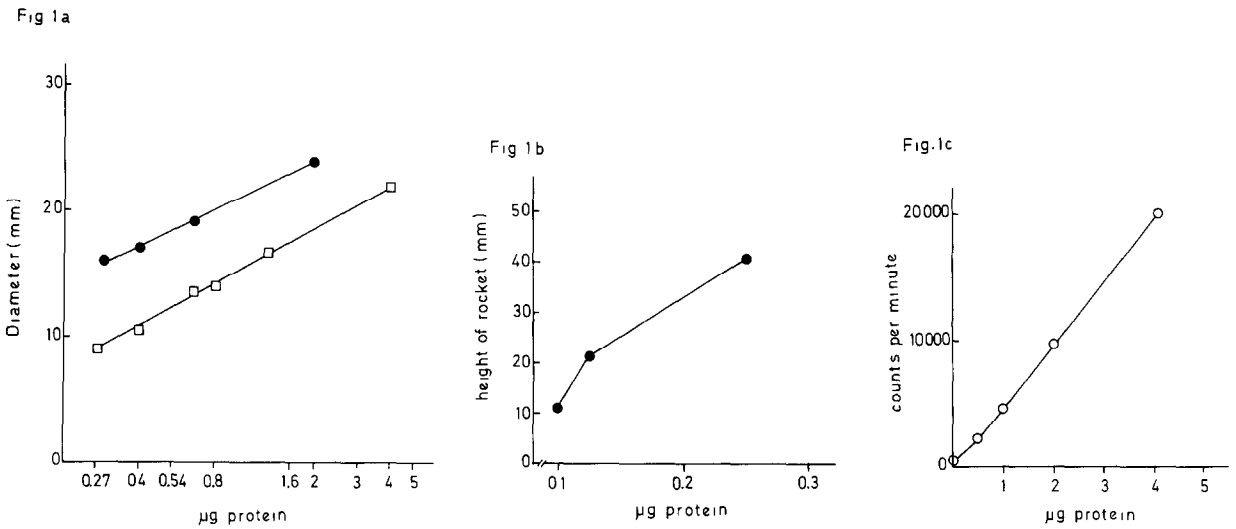

Fig.1. (a) Standard curve for: (a) Radial immunodiffusion – (□) glutathione transferases $A_2 + AC + C_2$; (●) glutathione transferases $B_2 + BL + L_2$. (b) Rocket immunoelectrophoresis – (●) glutathione transferases $B_2 + BL + L_2$. (c) Immunoblotting – (○) microsomal glutathione transferase. The experiments are described in section 2.

Table 1

Amounts of different glutathione transferases in rat liver microsomes

Glutathione transferase	μg/mg Microsomal protein[a]	Calculated contribution to microsomal activity[b]	
		Untreated	NEM-activated[c]
Microsomal	31 ± 0.6 (n = 7)	62 (36%)	930 (90%)
$A_2 + AC + C_2$	2.4 ± 0.6 (n = 4)	67 (39%)	67 (6%)
$B_2 + BL + L_2$	1.5 ± 0.1 (n = 3)	41 (24%)	41 (4%)
Total	35	170[d]	1038[e]

[a] Means ± SD from two separate experiments; n is the number of observations that were in the linear portions of standard curves. Transferases $A_2 + AC + C_2$ were measured with radial immunodiffusion. Transferases $B_2 + BL + L_2$ were also estimated by this method and rocket immunoelectrophoresis. Microsomal glutathione transferase was estimated by immunoblotting

[b] Specific activities (μmol 1-chloro-2,4-dinitrobenzene conjugated.min^{-1}.mg^{-1}) used were the mean for the A–C group, 28 and the B–L group, 27 (from [5]) and for unactivated and NEM-activated microsomal transferase 2 and 30, respectively (from [12,13])

[c] Activation with N-ethylmaleimide as in [11]

[d] Observed activity = 120; [e] Observed activity = 800

and is present in great excess over the cytosolic enzymes. The value of 3.1% for the microsomal enzyme agrees well with a purification factor of 36 (2.8% content) [12]. The value for the transferase BL family is somewhat underestimated because the sera have a lower titer with transferase L_2. Since all glutathione transferases appear to have different substrate specificities [1,5] the relative contribu-

tion of each form to the conjugation of any given substrate with glutathione by microsomes will depend on the nature of the compound. Activation of the microsomal enzyme by thiol reagents can, of course, also change the pattern.

In table 1 we have also calculated the relative contribution of the cytosolic and microsomal enzyme forms to the conjugation of 1-chloro-2,4-dinitrobenzene in microsomes before and after activation. As can be seen, activation shifts the relative contribution of the microsomal enzyme from 36% to 90%. When comparing the values obtained by calculation of the total activity using the contents and specific activities of the individual enzymes, reasonable agreement with the total measurable activity is found. The slightly higher total activity obtained by calculation might reflect the fact that some or all of the enzymes have higher activity in solubilized, isolated form than in situ in the microsomal membrane. This view is supported by the observation that solubilization of microsomes in Triton X-100 gives a slight increase in activity.

It is thus clear that microsomes possess the potential for catalyzing the conjugation of glutathione to a variety of compounds. The location of such activity in the endoplasmic reticulum might be advantageous for two reasons:

(i) Most substrates for glutathione transferases are lipophilic and are expected to dissolve extensively in membranes;
(ii) Electrophilic metabolites from the cytochrome P-450 system are generated in this membrane system and can be substrates for these enzymes (see [19]).

Work now performed in our laboratory demonstrates that the microsomal glutathione transferases have a broad substrate specificity [13], in analogy to the cytosolic enzymes [1].

The question as to whether the 'cytosolic' glutathione transferases are actually functionally localized on the endoplasmic reticulum cannot be answered conclusively at present. Evidence from different washing procedures [9,10] suggests that the presence of these enzymes in microsomes does not simply reflect cytosolic contamination. In addition, comparison with the level of the cytosolic marker lactate dehydrogenase in microsomes reveals that the 'cytosolic' glutathione transferases are present at a 10-times higher level than would be expected on the basis of contamination [9]. Furthermore, induction of the cytosolic activity up to 3–4-fold does not increase the microsomal activity [10]. This fact speaks against contamination and for the presence of defined amounts of the cytosolic enzymes in relationship to microsomal protein. The situation in mouse liver microsomes is the same. Authors in [20] have identified the soluble enzyme F_2 and F_3 as associated to the microsomal membrane by immunological and other methods. However, their work was not concerned with the microsomal glutathione transferase activatable by thiol reagents which is also present in mouse microsomes [21]. Evidence from immunoblotting experiments (not shown) reveals that a form in the mouse, which has the same M_r, crossreacts with antibodies against the rat enzyme. It is reasonable to assume from the similarity in activity after activation that the microsomal form is present in large excess, also in the mouse.

If the 'cytosolic' glutathione transferases are stable components of the endoplasmic reticulum in vivo, they may be found to differ slightly (e.g., in molecular mass or amino acid composition) from the soluble enzymes. Another possibility is that cytosolic glutathione transferases may bind to the endoplasmic reticulum under certain conditions and be released under other conditions, as is the case with hexokinase and mitochondria in rat brain [22]. We have observed that removal of ribosomes does not decrease microsomal glutathione transferase activity indicating that the microsomal 'cytosolic' glutathione transferases are probably not newly-synthesized proteins which have not yet been released into the cytoplasm.

We have here examined the major forms of cytosolic glutathione transferase, which, together with the microsomal enzyme, seem to account for all of the conjugating activity towards 1-chloro-2,4-dinitrobenzene found in microsomes (table 1). However, it is possible that small amounts of the minor soluble forms may also be present in microsomes.

The high level of the microsomal glutathione transferase suggests that, like the cytosolic transferase, it might also function as a binding protein. Another possible role for this enzyme is suggested by the finding [23–25] that there is a heat-labile factor in microsomes which can protect against lipid peroxidation. The microsomal glutathione

transferase might be involved here, since microsomal glutathione peroxidase activity towards cumene hydroperoxide can be activated 2-fold by *N*-ethylmaleimide [23]. We have found that the purified microsomal glutathione transferase has glutathione peroxidase activity towards cumene hydroperoxide which can be activated 10-fold by *N*-ethylmaleimide [13].

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