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Studies on the glutathione *S*-transferase activity associated with rat liver mitochondria

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The major proportion of rat liver glutathione *S*-transferase is cytosolic. Carefully washed mitochondria contain 0.25–0.47% of the cytosolic activity. Subfractionation of washed mitochondria using digitonin treatment revealed that glutathione *S*-transferase release did not parallel that of any of the mitochondrial marker enzymes. Glutathione *S*-transferase release paralleled that of lactate dehydrogenase, suggesting that these 'mitochondrial' activities are due to loosely bound cytoplasmic forms.

The glutathione *S*-transferases (GSTs) play a major role in the detoxification and excretion of many mutagenic, carcinogenic and pharmacologically active substances (Boyland & Chasseaud, 1969; Chasseaud, 1979; Glatt *et al.*, 1983). Although most studies on these enzymes have concentrated on the cytosolic forms (for review, see Jakoby, 1978), there have been reports of GST activity in microsomal and mitochondrial fractions (Wahlländer *et al.*, 1979; Kraus & Gross, 1979; Morgenstern *et al.*, 1980). The existence of a microsomal GST, activated by treatment with *N*-ethylmaleimide (in contrast with the cytosolic forms, which are not affected by *N*-ethylmaleimide under similar conditions), is well-established (Morgenstern *et al.*, 1979, 1980). This form has recently been purified and shown to be structurally and immunologically distinct from the cytosolic enzymes (Morgenstern *et al.*, 1982).

There is no such consensus with regard to the GST activity observed in mitochondrial fractions. Both Wahlländer *et al.* (1979) and Kraus (1980) reported that 7% of the total GST activity was mitochondrial, whereas Morgenstern *et al.* (1982) reported no enrichment of GST activity in mitochondrial fractions. As there is evidence for a mtiochondrial pool of glutathione and other glutathione-utilizing enzymes (Wahlländer *et al*, 1979; Kraus, 1980; Eklöw *et al.*, 1984; Beatrice *et al.*, 1984; Jocelyn & Cronshaw, 1984), it is important to establish whether this includes GST.

Abbreviations used: GST(s), glutathione *S*-transferase(s); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; CDNB, 1-chloro-2,4-dinitrobenzene.

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We have observed that washed mitochondria from rat liver have less than 0.5% of the total GST activity (C. M. Ryle & T. J. Mantle, unpublished work) and have therefore examined the nature of this residual activity. Our results suggest that this activity is due to cytosolic enzyme forms binding to the mitochondrial outer membrane.

Experimental

Materials

NAD^+, NADH, digitonin, Hepes and rotenone were obtained from Sigma. All other chemicals were obtained from BDH.

Preparation of mitochondria

Male Wistar rats (180–200g) were starved overnight before being killed by a blow on the head and exsanguinated. Livers were removed, weighed, rinsed in ice-cold 10mM-sodium phosphate/0.25M-sucrose/2mM-EDTA, pH7.2 (buffer A) and then homogenized in 4 vol. of the same buffer. All subsequent steps were conducted at 4°C. The homogenate was centrifuged at 800*g* for 8min. The resultant supernatant was centrifuged at 12000*g* for 12.5min, and the mitochondrial pellet obtained (M_1) was resuspended and washed in the required buffer. The supernatant from this centrifugation was referred to as the 'cytosolic fraction'.

Investigation of latent enzyme activity

A mitochondrial fraction that had been washed three times in buffer A was routinely used when assaying for release of enzyme activity by digitonin

and when testing for latent enzyme activity. In one experiment a mitochondrial fraction was prepared as described above, except that the buffer used throughout was 67mM-sodium phosphate/0.2M-KCl/2mM-EDTA, pH7.2 (buffer B).

Submitochondrial fractionation

The distribution of enzyme activities within the mitochondrial subfractions was investigated by studying their release by digitonin (Schnaitman *et al.*, 1967; see also Greenawalt, 1974). The method was essentially as described by Ryle & Tipton (1981). Briefly, freshly prepared mitochondria that had been washed three times in buffer A were finally resuspended in buffer A or in 4.0% (w/v) mannitol/2.4% (w/v) sucrose/0.048% Hepes/2mM-EDTA, adjusted to pH7.4 with KOH (buffer C), to give a final protein concentration of between 6 and 7mg/ml. Aliquots of a freshly prepared digitonin solution (5mg/ml) were added to samples of the mitochondrial suspension to give the desired concentration, and the mixtures were left on ice for 30min with occasional shaking. Samples of the mixtures were retained for assay, and the remainder were centrifuged for 3min in a Mechanika Precyzyjna 320a Minifuge. The pellets obtained after digitonin treatment was resuspended in buffer A or buffer C containing 0.4% (v/v) Triton X-100.

Assay methods

Lactate dehydrogenase, glutamate dehydrogenase, monoamine oxidase, sulphite:cytochrome *c* reductase and succinate dehydrogenase were assayed as described previously (Ryle & Tipton, 1981). GST activity was assayed as described by Habig *et al.* (1974), with CDNB as substrate. Protein was determined by the method of Markwell *et al.* (1978), with bovine serum albumin as a standard. To investigate latent enzyme activity, mitochondria were prepared and washed with buffer A as described above. After three washes the mitochondrial pellet was resuspended in either buffer A or buffer C, left on ice for 90min to mimic the control treatment for the digitonin-release experiments and then centrifuged at 12500 rev./min (12000*g*) for 3min in a Mechanika Precyzyjna 320a Minifuge. To obtain the total enzyme activity in the resultant pellets, they were resuspended in buffer A or in buffer C containing 0.4% (v/v) Triton X-100. When assaying for latent enzyme activity the buffer used was supplemented with 0.15M- sucrose, and 20μl of rotenone (100μg/ml in ethanol) was added to the assay mixture. After addition of all assay constituents, 10μl of 0.4% (v/v) Triton X-100 was added, followed by 10μl of 4% (v/v) Triton X-100. The final assay volume was 2.2ml.

Results and discussion

Table 1 shows the GST activity obtained in the mitochondrial fraction after repeated washings in various buffers. Although it was not possible, with any buffer used, to remove completely GST activity observed in this fraction, the amount of activity remaining after five washes was only 0.25–0.47% of the activity obtained in the supernatant fraction. The activity in the mitochondrial fraction appeared to reach a plateau after three washes, and this observation agrees qualitatively with the findings of Kraus & Gross (1979). However, as similar results were obtained when lactate dehydrogenase was assayed in the washed mitochondrial fractions (C. M. Ryle & T. J. Mantle, unpublished work), the submitochondrial location of the GST activity in washed mitochondria was investigated further.

Figs. 1 and 2 show the effect of increasing concentrations of digitonin on the release of various enzymes from the mitochondrial fraction. When three-times-washed mitochondria were incubated in buffer C, the release of GST did not parallel that of any of the mitochondrial marker enzymes, but was very similar to that obtained for lactate dehydrogenase (see Fig. 1). When incubation of the mitochondria was performed in buffer A, the release of GST activity again differed markedly from the release pattern obtained for the marker enzymes (Fig. 2). However, when using this buffer, although there was an initial parallel release of GST and lactate dehydrogenase at low digitonin concentrations, there was a slight but significant deviation at higher concentrations. This experiment has been repeated twice, and the range of plateau values for GST and lactate dehydrogenase at a digitonin/protein ratio of 0.16 was 57–69% and 86–91% respectively.

Examination of Figs. 1 and 2 shows that digitonin releases GST from the mitochondrial fraction in two distinct phases. Approximately half the GST activity associated with mitochondria washed in buffer A is released in the absence of digitonin simply by resuspending the mitochondria in buffer C (see Fig. 1). A similar fraction is released at very low digitonin concentrations when buffer A is used to resuspend the mitochondrial pellet (see Fig. 2). The interaction between the mitochondria and this easily released fraction of GST does not seem to be of an ionic nature, as washing mitochondrial fractions with buffer supplemented with 0.2M-KCl (buffer B) does not result in a decrease in GST activity in the mitochondrial pellet (see Table 1). Furthermore, when mitochondria were prepared and washed in buffer B, resuspended in buffer A and then incubated with low concentrations of digitonin,

Table 1. *Effect of repeated washing on the activity of GST in mitochondria*

A mitochondrial fraction (M_1) from six rat livers was prepared as described in the text. The GST activity in the cytosolic fraction was 22100 units. The M_1 fraction was then divided into three equal fractions, which were assayed for GST activity and then washed in the buffers shown. The recovery of GST activity after each wash was >85%. One unit of enzyme activity is defined as that which, under the assay conditions described in the text, resulted in a change of $1A_{340}$ unit/min.

	GST activity					
Buffer ...	10mM-Sodium phosphate/ 0.25M-sucrose/ 2mM-EDTA, pH7.2 (A)		67mM-Sodium phosphate/ 0.2M-KCl (pH7.2)/ 2mM-EDTA (B)		67mM-Sodium phosphate (pH7.2)/2mM-EDTA	
Fraction	(units)	(%)	(units)	(%)	(units)	(%)
Mitochondrial pellet (M_1)	181	100	147	100	158	100
After wash no.						
1	64	35.4	63	43	54	34
2	59	32.6	52	35	42	27
3	43	24	35	24	31	20
4	38	21	34	23	29	18
5	35	19	19	13	23	15

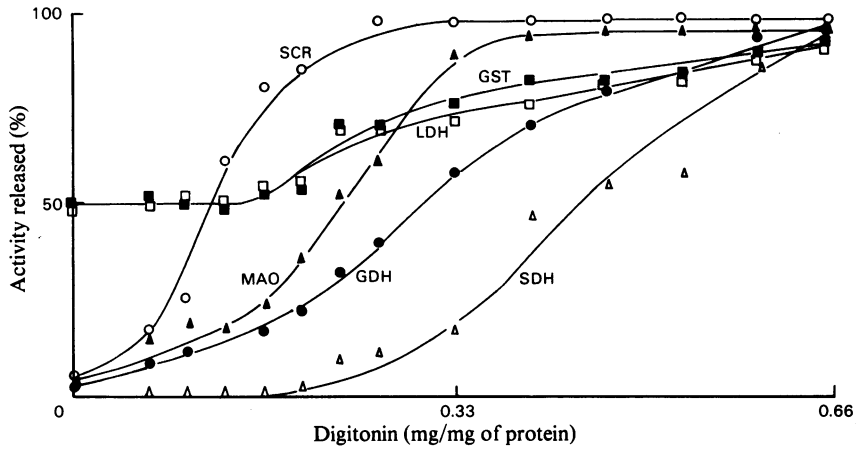

Fig. 1. *Effect of digitonin concentration on enzyme release from mitochondria in buffer C*

The release of the marker enzymes monoamine oxidase (MAO, ▲), sulphite:cytochrome *c* reductase (SCR, ○), glutamate dehydrogenase (GDH, ●) and succinate dehydrogenase (SDH, △) and of GST (■) and lactate dehydrogenase (LDH, □) are shown for three-times-washed mitochondria.

the patterns of release of lactate dehydrogenase, glutamate dehydrogenase and GST were similar to those shown in Fig. 2 (results not shown). These results suggest that this fraction of GST activity associated with the washed mitochondrial fraction is due to cytosolic forms binding loosely to the mitochondrial outer membrane.

The second phase of release of GST activity essentially parallels that of glutamate dehydrogenase and begins at a digitonin/protein ratio of approx. 0.25 in either buffer A or C. This accounts for 40–50% of the GST activity observed in the washed mitochondrial pellet. The location of this activity is more difficult to assign and may be due to (i) GST(s) located in the mitochondrial matrix or (ii) a second fraction of cytosolic GST activity bound more tightly to the outer mitochondrial membranes. It is difficult to distinguish between these two possibilities. If it is assumed that lactate dehydrogenase is found exclusively in the cytosol, the results shown in Fig. 1 suggest that all the GST activity associated with the mitochondrial fraction is cytosolic in origin. However, the dissimilar release curves obtained for GST and lactate dehydrogenase at higher concentrations of digitonin in buffer A could result from release of a fraction of the GST from the mitochondrial matrix.

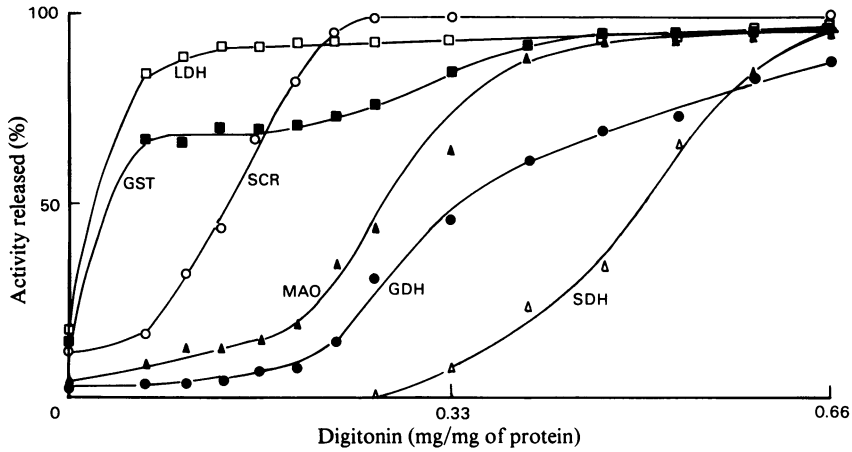

Fig. 2. *Effect of digitonin concentration enzyme release from mitochondria in buffer A*
For details, see the legend to Fig. 1.

In an attempt to determine whether, in buffer A, the second phase of GST release by digitonin was release of GST located in the matrix, a washed mitochondrial fraction was assayed in the presence of two concentrations of Triton X-100 in an attempt to observe latency. Treatment of mitochondria with a low concentration of Triton X-100 (0.0013%) caused no increase in either GST or lactate dehydrogenase activity, whereas the activity of glutamate dehydrogenase increased by approx. 60%. At a higher concentration of Triton X-100 (0.014%) the activity of both GST and lactate dehydrogenase increased by 90–100%, whereas an approx. 5-fold increase in glutamate dehydrogenase activity was observed. Neither GST nor lactate dehydrogenase activity displayed the same 'latency' as glutamate dehydrogenase, suggesting that GST is not located in the mitochondrial matrix.

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References

Beatrice, M. C., Stiers, D. L. & Pfeiffer, D. R. (1984) *J. Biol. Chem.* **259**, 1279–1287

Boyland, E. & Chasseaud, L. F. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol.* **32**, 173–219

Chasseaud, L. F. (1979) *Adv. Cancer Res.* **29**, 175–274

Eklöw, L., Moldeus, P. & Orrenius, S. (1984) *Eur. J. Biochem.* **138**, 459–463

Glatt, H., Friendberg, T., Grover, P. L., Sims, P. & Oesch, F. (1983) *Cancer Res.* **43**, 5713–5717

Greenawalt, J. W. (1974) *Methods Enzymol.* **31A**, 310–323

Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139

Jakoby, W. B. (1978) *Adv. Enzymol.* **46**, 383–414

Jocelyn, P. C. & Cronshaw, A. D. (1984) *Biochim. Biophys. Acta* **797**, 203–211

Kraus, P. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 9–15

Kraus, P. & Gross, B. (1979) *Enzyme* **24**, 205–208

Markwell, M., Hass, S. M., Buker, L. & Tolbert, N. (1978) *Anal. Biochem.* **87**, 206–210

Morgenstern, R., DePierre, J. W. & Ernster, L. (1979) *Biochem. Biophys. Res. Commun.* **87**(3), 657–663

Morgenstern, R., Meijer, J., DePierre, J. W. & Ernster, L. (1980) *Eur. J. Biochem.* **104**, 167–174

Morgenstern, R., Guthenberg, C. & DePierre, J. W. (1982) *Eur. J. Biochem.* **128**, 243–248

Ryle, C. M. & Tipton, K. F. (1981) *Biochem. J.* **197**, 715–720

Schnaitman, L., Erwin, V. G. & Greenawalt, J. W. (1967) *J. Cell Biol.* **32**, 719–735

Wahlländer, A., Soboll, S. & Sies, H. (1979) *FEBS Lett.* **97**(1), 138–140