

A novel glutathione transferase (13–13) isolated from the matrix of rat liver mitochondria having structural similarity to class Theta enzymes

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A rat liver mitochondrial-matrix fraction was prepared and shown to have 1-chloro-2,4-dinitrobenzene(CDNB)-metabolizing glutathione transferase (GST) activity. Further fractionation by sequential gel filtration, isoelectric focusing or chromatofocusing and hydroxyapatite chromatography yielded three GSTs of pI 9.3, 8.9 and 7.5, none of which bound to a GSH-agarose affinity matrix. Most of the activity was associated with the pI-9.3 form, which was selected for further study. Its activity was tested with the following potential substrates in addition to CDNB: 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride, *trans*-4-phenylbut-3-en-2-one, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, ethacrynic acid, menaphthyl sulphate, cumene hydroperoxide, linoleic acid hydroperoxide and 4-hydroxynon-2-enal. Appreciable activity was obtained only with CDNB and ethacrynic acid (82 and 26 $\mu\text{mol}/\text{min}$ per mg of protein respectively). The apparent K_m for GSH, using 1 mM-CDNB, was 1.9 mM. The enzyme is a dimer of subunit M_r 26500. It has a free *N*-terminus, which has enabled the first 33 amino acids to be sequenced. This portion of primary structure has a sequence in common with members of the Theta class of GSTs (eg. 36% identity with subunit 12) and also a sequence which might function as a mitochondrial import signal. It is novel and has been named 'GST 13–13'.

INTRODUCTION

Glutathione transferases (GSTs) (EC 2.5.1.18) isolated from the soluble supernatant and microsomal fractions have been studied in detail. Those from the soluble supernatant are dimers, the subunits of which are members of four multigene families referred to as Alpha, Mu, Pi and Theta (Mannervik *et al.*, 1985; Meyer *et al.*, 1991). They have several functions, one being the detoxication of xenobiotic electrophiles, including those derived from the metabolism of drugs, carcinogens and environmental chemicals and another being protection against the reactive products of free-radical damage to membrane lipids (reviewed by Ketterer *et al.*, 1988). The only microsomal GST so far isolated has a number of enzymic properties in common with the soluble enzymes, but is structurally unrelated (Morgenstern *et al.*, 1982).

Less is known about soluble mitochondrial GSTs. GST activity has been observed in mitochondrial preparations (Wahländer *et al.*, 1979; Jocelyn & Cronshaw, 1985; Botti *et al.*, 1989) and the purification and partial characterization of three mitochondrial GSTs has been reported by Kraus (1980). However, Ryle & Mantle (1984) have suggested that these activities may be the result of cytoplasmic contamination. GST activity in the mitochondria, like that of the cytoplasm, might be present to protect against genotoxic and cytotoxic electrophiles. These could enter from the extramitochondrial cytoplasm (Wunderlich *et al.*, 1970, 1971; Wilkinson *et al.*, 1975; Egilsson *et al.*, 1979; Allen & Coombs, 1980; Backer & Weinstein, 1980; Myers *et al.*, 1988; Smith *et al.*, 1989), be produced within the mitochondria by the activity of mitochondrial cytochrome *P*-450 species (Niranjan *et al.*, 1984) or might result from the decomposition of lipid peroxides produced during respiration (Hruszkewycz, 1987; Hruszkewycz & Bergtold, 1987).

Since the cytosolic GSTs lack mitochondrial-import sequences, it is likely that any truly mitochondrial GST would differ from the cytoplasmic enzymes so far characterized. The present paper

describes the isolation from the mitochondrial matrix of such a novel GST. Some of its properties as a protein and an enzyme have been determined, and its possible function in the mitochondrion has been assessed.

EXPERIMENTAL

Isolation of rat liver mitoplasts

Male Wistar rats (200–300 g) were killed by cervical dislocation, their livers removed, minced and homogenized (2:1, v/w) in buffer A [70 mM-sucrose/220 mM-mannitol/BSA (defatted)(0.5 mg/ml)/2 mM-Hepes/KOH, pH 7.4]. Mitochondria were isolated by differential centrifugation three times through buffer A (Greenawalt, 1974). The purity of the preparation was assessed by electron microscopy as follows. The mitochondrial pellet (0.5 ml) was resuspended and fixed with 2% glutaraldehyde and 1% OsO₄ in 0.1 M-sodium cacodylate, pH 7, before dehydration in ethanol and propylene oxide. The pellet, fixed and dried in this manner, was embedded in Araldite resin and sections were cut for electron microscopy.

The mitochondrial outer membrane and the contents of the intermembrane space were removed by osmotic shock in a volume of buffer A/water (1:3, v/v) corresponding to half that of the original homogenate (Botti *et al.*, 1989). Removal of the intermembrane space and the purity of the resulting mitoplasts (inner membrane and matrix) were monitored by assaying fumarase activity as a matrix marker (Tolbert, 1974) and adenylate kinase activity as an indication of contamination arising from the intermembrane space (Sottocasa *et al.*, 1967). Finally the mitoplasts were washed with 0.3 M-KCl to remove loosely bound proteins, including GSTs bound non-specifically.

Purification of matrix GST

Mitoplasts purified from the livers of six rats were sonicated in buffer B [10 mM-sodium phosphate (pH 7.0)/150 mM-KCl/2 mM-

dithiothreitol/3 mM- β -mercaptoethanol/25 μ M-phenylmethanesulphonyl fluoride] and the sonicated material was centrifuged at 144000 *g* to sediment membrane fragments. The supernatant was subjected to gel filtration using a Sephacryl S-300 column (300 mm \times 10 mm) in buffer B. Fractions with CDNB-metabolizing GST activity were pooled and concentrated by centrifugal ultrafiltration using a Centriprep-10 (Amicon, Danvers, MA, U.S.A.). The sample was then transferred into buffer C [25 mM-diethanolamine (pH 9.5)/7% (v/v) glycerol/5 mM- β -mercaptoethanol] and applied to a Mono P (HR 5/20) chromatofocusing column equilibrated at ambient temperature in buffer C using a Pharmacia-LKB (Uppsala, Sweden) f.p.l.c. apparatus). The column was developed at 0.3 ml/min with Polybuffer 96 adjusted to pH 6.0 with HCl. In a larger-scale preparation this step was replaced by column isoelectric focusing over the pH range 3.5–11 (Beale *et al.*, 1983). Three active fractions were obtained by each method. Each was transferred into buffer D [5 mM-sodium phosphate/10 μ M-CaCl₂/10 mM- β -mercaptoethanol/5 mM-dithiothreitol/5% (v/v) glycerol] and further purified by hydroxyapatite chromatography using a 100 mm \times 7.8 mm Bio-Gel HPHT column (Bio-Rad, Richmond, CA, U.S.A.) operated at 0.3 ml/min by the f.p.l.c. and developed with a linear gradient (30 ml) of 5–400 mM-sodium phosphate. Final purification of the major form was achieved by reverse-phase h.p.l.c. (Kispert *et al.*, 1989). The GST protein was identified after h.p.l.c. by a reconstitution technique which has so far proved successful with other rat cytosolic GSTs (J. M. Harris, D. J. Meyer, L. E. Lalor, B. Coles & B. Ketterer, unpublished work). Briefly, samples from h.p.l.c. were mixed 1:1 with AnalaR-grade glycerol, incubated at room temperature for 2 min, then diluted with 0.1 M-sodium phosphate, pH 6.8, containing 1 mM-CDNB and 1 mM-GSH and assayed at 37 °C (Habig *et al.*, 1974); 1 unit of activity is defined as 1 μ mol of substrate converted/min. The purification is summarized in Table 1.

Parallel with the mitochondrial GST preparation, a portion of liver was used to prepare cytosolic GSTs by GSH-agarose affinity chromatography as described by Vander Jagt *et al.* (1985). Protein was measured by its absorbance at 280 nm or by the method of Bradford (1976).

Characterization of major matrix GST

GST and peroxidase activities were assayed as described by Habig *et al.*, (1974) and Prohaska & Ganther (1976) respectively. Linoleate hydroperoxide was synthesized as described by O'Brien (1969). The K_m for GSH was measured using 1 mM-CDNB as second substrate.

Immunoreactivity was determined by Western blotting (Towbin *et al.*, 1979) using polyclonal antibodies raised to rat and human GSTs.

Table 1. Purification of GST 13–13 from mitochondria

Step	Total activity (units)	Specific activity (units/mg of protein)	Purification	Yield (%)
Sonicated mitoplasts	15.12	0.42	1	100
Chromatofocusing	5.88	4.90	11.7	38.7
Hydroxyapatite	0.57	28.50	67.9	3.8
Reverse-phase h.p.l.c.	0.49	82.10	123.3	3.2

* The enzyme activity with CDNB was measured at 37 °C as described by Habig *et al.* (1974); 1 unit is defined as 1 μ mol of substrate converted/min.

SDS/PAGE was carried out using a 12%-(w/v)-acrylamide resolving gel with 2% (w/v) bisacrylamide (Laemmli, 1970).

N-Terminal amino acid sequencing was carried out on material from chromatofocusing by using an Applied Biosystems 470A sequencer.

RESULTS

GST isolation and structural characterization

Electron microscopy showed the mitochondrial preparation to be homogeneous, with negligible contamination from other organelles (Fig. 1*a*). Most of the mitochondria were intact and had clear contracted cristae suggestive of State 4 respiration (Fig. 1*b*). Osmotic shock of the mitochondria and subsequent harvesting by centrifugation yielded a supernatant containing less than 5% of the total mitochondrial fumarase activity. Conversely, no adenylate kinase activity could be detected in the mitoplast

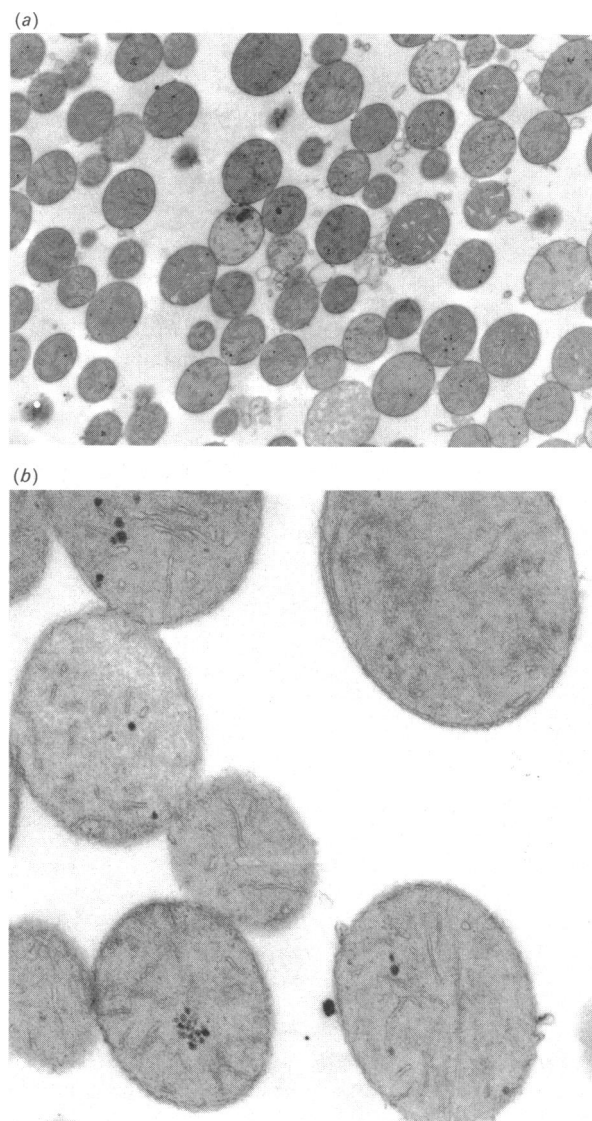


Fig. 1. Electron micrographs of mitochondria

Mitochondria prepared by the method of Greenawalt (1974) were fixed in 2% glutaraldehyde and 1% OsO₄, dehydrated in ethanol and propylene oxide and embedded in Araldite resin before electron microscopy as described in the text. Magnifications: (a) \times 2700; (b) \times 9000.

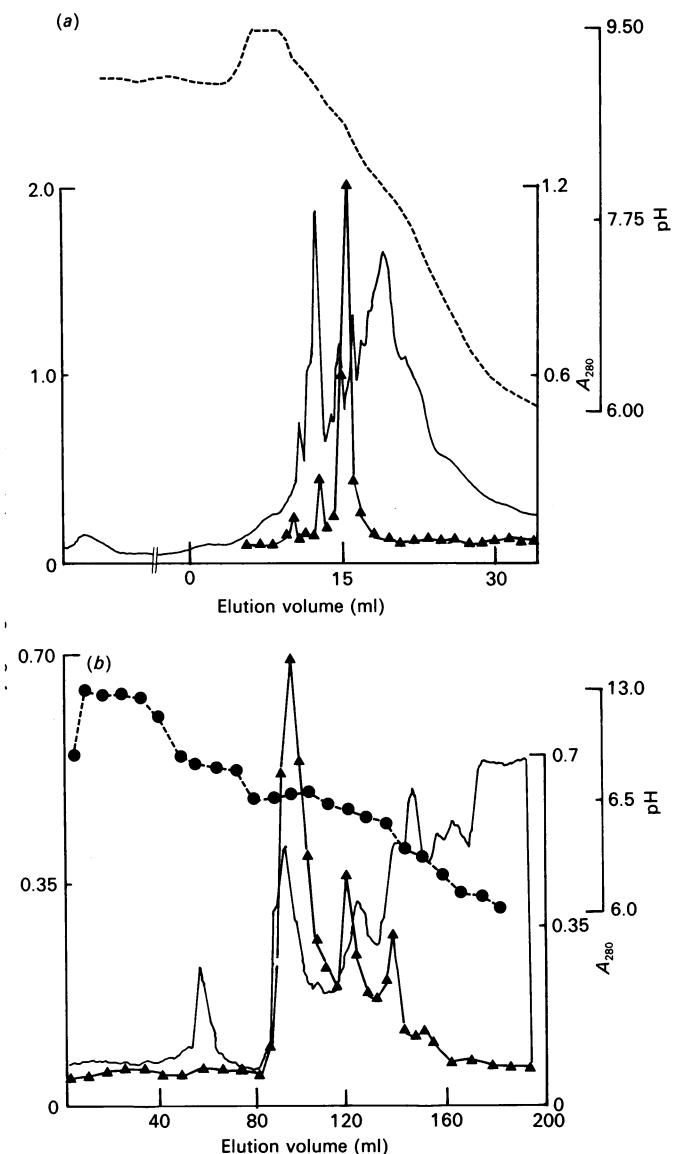


Fig. 2. Analysis of mitochondrial-matrix GSTs by f.p.l.c. chromatofocusing and isoelectric focusing

Active fractions from gel filtration of sonicated mitoplasts were subjected to f.p.l.c. chromatofocusing over the pH range 9.5–6.0 (a) or column isoelectric focusing in a sucrose density gradient over the range 11.0–3.5 (b). Fractions were monitored for CDNB-conjugating activity (\blacktriangle), A_{280} (—) and pH (----) as described in the text.

fraction after sonication. The matrix preparation showed GST activity with CDNB of 0.42 unit/mg of total protein, which was approx. 20% of that of the cytosol from the same livers (1.8 units/mg of total protein). Gel filtration gave a single peak of GST activity that was eluted with an M_r of approx. 56000 (results not shown). A sample of the pooled CDNB-metabolizing activity from the gel filtration was tested for binding to a GSH-agarose affinity matrix. No CDNB activity or inactive GST protein binding could be detected, in contrast with the cytosol fraction, where 95% of the CDNB-metabolizing activity bound. Further purification was achieved by column isoelectric focusing (Fig. 2a) or chromatofocusing (Fig. 2b), which gave three catalytically active fractions of pI 9.3 (major GST CDNB-metabolizing activity), 8.9 and 7.5. The most active fraction (pI 9.3) yielded a single peak of activity on hydroxyapatite chromatography which was eluted with 290 mM-sodium phosphate (Fig. 3). This fraction separated into two major protein peaks on

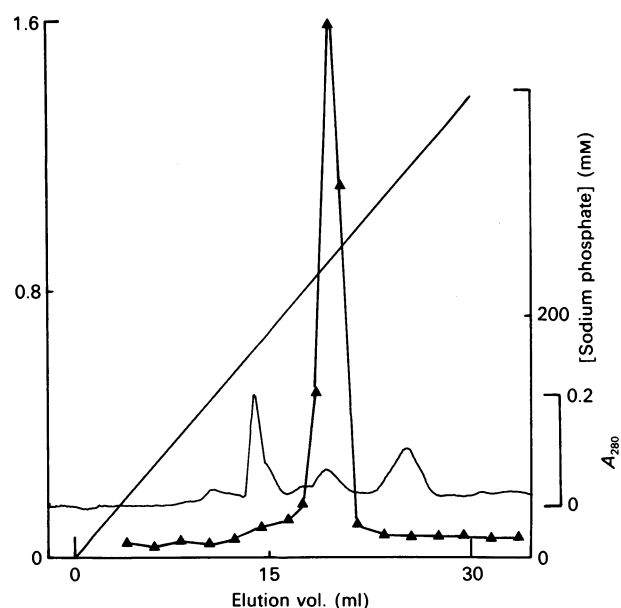


Fig. 3. Analysis of mitochondrial GST by hydroxyapatite f.p.l.c.

The major active fraction from chromatofocusing or isoelectric focusing was transferred into buffer D, applied to an hydroxyapatite f.p.l.c. column equilibrated in this buffer and eluted with a linear sodium phosphate gradient (straight diagonal line) as described in the text. Fractions were monitored for CDNB-conjugating activity (\blacktriangle) and A_{280} (—).

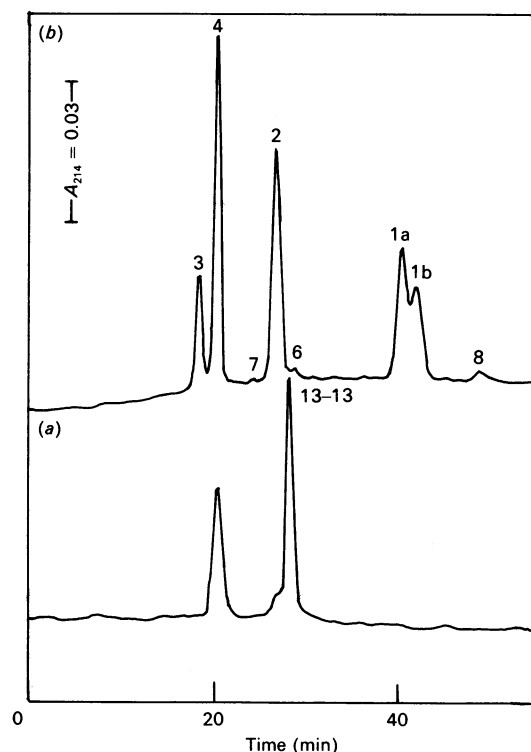


Fig. 4. Analysis of mitochondrial and cytosolic GSTs by reverse-phase h.p.l.c.

The fraction with highest CDNB-conjugating activity from the hydroxyapatite f.p.l.c. was further purified by h.p.l.c. as described in the text. The sample applied (a) is that obtained for 8 mg of total matrix soluble protein and is compared with cytosolic GSTs (b) prepared in parallel by GSH affinity chromatography from 4 mg of total cytosolic protein; GST subunits are labelled.

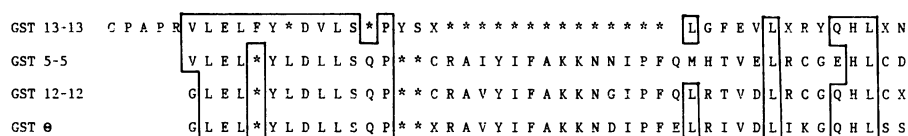


Fig. 5. Comparison of *N*-terminal sequences from rat GST subunits 13, 5, 12 and human GST ̸.

GST subunit 13 (15 μ g) finally purified by h.p.l.c. was subjected to *N*-terminal sequencing as described in the text. The sequence is compared with those for subunit 5, 12 and ̸ (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991).

reverse-phase h.p.l.c. (Fig. 4). Only the later-eluted peak yielded GSH transferase activity towards CDNB upon reconstitution. Note that, on chromatofocusing f.p.l.c., the major peak of CDNB activity was eluted with a lower apparent pI than with the isoelectric focusing; however, it behaved in a similar way to the isoelectric-focusing fraction on further purification. Amino acid sequencing identified the first 33 residues of the purified protein, which differ from all previously characterized GSTs, but show similarity to GSTs of the Theta class, particularly over residues 6–16 (Fig. 5). On SDS/PAGE/Western blotting the h.p.l.c. purified GST migrated as a single band with an apparent M_r of 26 500 (Yb) and gave a faint cross-reaction with an anti-[1–1 and 2–2 (Alpha class) GST] polyclonal antibody; however, no cross-reaction was detected with antisera raised to GST 5–5 or anti-(Mu-class-GST) anti-(Pi-class) GST antibodies (not shown).

The GST isolated is structurally distinct and we term it 'GST 13–13'.

Activity of GST 13–13

GST 13–13 partially purified by hydroxyapatite f.p.l.c. had a specific activity of 82 units/mg with CDNB and 26 units/mg with ethacrynic acid. When assayed with 1 mM-CDNB the enzyme had an apparent K_m for GSH of 1.9 mM at pH 6.5. Surprisingly the enzyme showed no GSH peroxidase activity with the model organic hydroperoxides or H_2O_2 and no catalytic activity was detected with a spectrum of substrates diagnostic for cytosolic GSTs, namely 1,2-dichloro-4-nitrobenzene, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, 4-hydroxynon-2-enal, 2-menaphthyl sulphate, *p*-nitrobenzyl chloride or *trans*-4-phenylbut-3-en-2-one.

DISCUSSION

The present study confirms the existence of a matrix-located mitochondrial GST. Since this is not a protein encoded by the mitochondrial genome (Anderson *et al.*, 1981), it must be synthesized in the cytoplasm. How then does it cross the mitochondrial outer and inner membranes to enter the matrix? Most proteins which locate within the mitochondrion have *N*-terminal presequences of 20–30 amino acids that do not occur in the mature form isolated from the mitochondria, having been cleaved by a matrix metalloproteinase (Pfanter & Neupert, 1987). Presequences are very heterogeneous, but all seem to consist of a number of basic amino acids occurring at fairly regular intervals and separated by uncharged amino acid residues, among which the hydroxylated amino acids threonine and serine are common. Such presequences have the capacity to form an amphiphilic α -helix which assists penetration of phospholipid bilayers (Roise *et al.*, 1988). The GST described here may have had an *N*-terminal extension which was cleaved on entering the mitochondrion; however, it is also possible that the structure of the mature catalytically active protein may be sufficient to allow mitochondrial import. When the *N*-terminus of GST 13–13 is aligned with related cytosolic sequences (Fig. 5), it is evident that the enzyme is extended by five amino acids, including an arginine

residue at position 5, which is conserved among mitochondrial presequences. The addition of the extra five amino acids makes the *N*-terminus of GST 13–13 both more basic and more amphiphilic than cytosolic GSTs. Although this extra portion of the *N*-terminus is considerably shorter than most presequences, it is possible that it alone enables GST 13–13 to be imported into the mitochondrion.

Cytosolic GSTs in mammals have distinct tissue distributions (Ketterer *et al.*, 1988). Within the cell there are apparently differences in the distribution of GSTs among the subcellular organelles, including the nucleus (Tan *et al.*, 1988), microsomes (Morgenstern *et al.*, 1982) and the mitochondrial outer membrane (Morgenstern *et al.*, 1984; Nishino & Ito, 1990).

Although so far it has only been found in the mitochondrial matrix, GST 13–13 is similar to cytosolic enzymes in that it is soluble and has a dimeric structure composed of subunits of M_r 26 500. It shares with the Theta class enzymes [5, 12, ̸ (Meyer *et al.*, 1991); Yrs (12) (Hiratsuka *et al.*, 1990)] a failure to bind to the GSH-agarose affinity matrix and a K_m for GSH above 1 mM. It also has a similar retention time on reverse-phase h.p.l.c. and some *N*-terminal sequence in common. However no cross-reactivity between GST 13–13 and antisera raised to Theta class enzymes is observed.

GST 13–13 differs from the GST that was isolated by Kraus (1980) and considered to be responsible for most of the matrix CDNB-metabolizing activity. This GST has a pI of 7.1–7.4, a K_m for GSH of 0.3 mM, and a specific activity of 3.0 units/mg for CDNB, whereas GST 13–13 has a pI of 9.3, a K_m of 1.9 mM and specific activity for CDNB of 82 units/mg. These differences could be due to Kraus' (1980) use of freezing and thawing to rupture mitochondria, a process which may have caused denaturation.

It is noteworthy that both GST 13–13 and 5–5 have structural similarities to the dichloromethane dehalogenase of *Methylobacterium sp.* (La Roche & Leisinger, 1990). This primitive pigmented eubacterium is similar to the putative ancestor of mitochondria (Woese, 1981), suggesting that there might be an evolutionary relationship between the bacterial, mitochondrial and Theta class cytosolic GSTs of eukaryotes.

So far the known substrates for GST 13–13, as for bacterial GSTs, are remarkably few (Di Ilio *et al.*, 1988; Ilizuka *et al.*, 1989). It will be of interest to extend our search for possible substrates. The work of Botti *et al.* (1989) suggests that ethylene dibromide may be a substrate.

The CDNB-conjugating activity of GST 13–13 probably contributes to the depletion of mitochondrial GSH by CDNB reported by Jocelyn & Cronshaw (1985); similarly the high specific activity with ethacrynic acid may contribute to the GSH depletion of mitochondria reported by Meredith & Reed (1982); it may also be an important factor in the reported ethacrynic acid enhancement of melphalan toxicity (Clapper *et al.*, 1989; Hansson *et al.*, 1990) and in current phase-two and -three clinical trials of a combined ethacrynic acid/thiotepa drug regimen.

The lack of activity towards 4-hydroxynonanal is rather surprising, considering that most GSTs have large or at least significant activity (Ålin *et al.*, 1985), and in view of the large

quantities of superoxide anion produced in the mitochondrion with consequent lipid peroxidation and the production of toxic hydroxyalkenals (Hruszkewycz, 1987).

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