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# Active site solvation contributes significantly to inactivation of the glutathione-*S*-transferases (GST)

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The glutathione-*S*-transferases (GST), a ubiquitous group of enzymes which catalyse the conjugation of glutathione (GSH) with electrophilic substrates, play a major role in xenobiotic metabolism and detoxication *in vivo*. An important aspect of GST chemistry is the fact that the enzymes are inactivated, both reversibly and irreversibly, by adding non-substrate ligands (e.g. bilirubin) or highly reactive substrates/GSH-cosubstrate complexes [1].

A third type of inhibition which does not appear to have been adequately characterized occurs when a concentrated stock solution of GST is diluted in buffer alone. This spontaneous inhibition, of which several examples appear (without comment) in the literature [2, 3], follows pseudo first-order kinetics with rate constant equal to $2.5 \times 10^{-3}$/sec (pH 6.5, 25°) and does not proceed to 100% inactivation.

In the present paper we describe both spontaneous dilutional inactivation of GST $\pi$ and inactivation induced by the non-substrate ligand microperoxidase-8 (MP-8), a monomeric aqueous phase model for ferriprotoporphyrin IX in its reactions with heme-binding proteins [4]; the GST co-substrate, 1-chloro-2,4-dinitrobenzene (CDNB); and the xenobiotic pre-carcinogen ethylene dibromide (EDB). A common origin for the inactivation, namely active site solvation, is suggested on the basis of an invariant inactivation rate constant.

## *Materials and methods*

Glutathione *S*-transferase $\pi$ was prepared from human placenta in our laboratory by the method of Radulovic and Kulkarni [5]. The final product, which ran as a single band on SDS–PAGE electrophoresis was divided into 0.5 $cm^3$ aliquots and stored at 77°K without loss of activity. Human placental GST $\pi$ and rat liver GST mix were obtained from the Sigma Chemical Co. (St Louis, MO), and were dialysed overnight at 4° prior to use to remove traces of reduced glutathione (GSH). Rat liver GST 3–4 isoenzyme was purified by chromatofocusing; purity was assessed by elution profiles, specific activity toward different electrophilic substrates and gel electrophoresis. The product was equivalent to that reported by Mannervik and Jensson [6]. Microperoxidase-8 (MP-8) was prepared by sequential peptic and tryptic hydrolysis of horse heart cytochrome *c* using the optimized HPLC procedure recently reported [7], the purity of the hemepeptide was estimated >99.5%. Buffers used were phosphate, $\mu = 0.1$, pH 6.5. In all cases buffers

Table 1. Pseudo first-order rate constant ($k_{obs}$) for the irreversible inactivation of a range of Glutathione-*S*-transferases (GST)

| Inactivating agent | GST isoenzyme | Mean $k_{obs}$ (sec) | Reference |
|---|---|---|---|
| CDNB (1 mM) | Human placental $\pi$ | $1.5 \times 10^{-3}$ | [2: Fig. 2] |
| Solvent (1 mM EDTA), 30° | Human placental $\pi$ | $0.7 \times 10^{-3}$ | [3: Fig. 3] |
| CDNB (1 mM EDTA), 30° | Human placental $\pi$ | $1.2 \times 10^{-3}$ | [3: Fig. 3] |
| CTX (1 mM EDTA), 30° | Human placental $\pi$ | $0.5 \times 10^{-3}$ | [3: Fig. 3] |
| Solvent | Human placental $\pi$ (Sigma) | $2.5 \times 10^{-3}$ | [This work] |
| CDNB (1 mM) | Human placental $\pi$ (Sigma) | $1.7 \times 10^{-3}$ | [This work] |
| Solvent | Human placental $\pi$ (this prep) | $2.7 \times 10^{-3}$ | [This work] |
| CDNB (0.8 mM) | Human placental $\pi$ (this prep) | $1.7 \times 10^{-3}$ | [This work] |
| CDNB (1.2 mM) | Human placental $\pi$ (this prep) | $1.6 \times 10^{-3}$ | [This work] |
| MP-8 (0.4 $\mu$M)* | Human placental $\pi$ (this prep) | $2.2 \times 10^{-3}$ | [This work] |
| CDNB (0.5 mM), 37° | Erythrocyte | $10.5 \times 10^{-3}$ | [10: Fig. 1] |
| CDNB (1.2 mM) | Rat liver GST mix (Sigma) | $2.5 \times 10^{-3}$ | [This work] |
| EDB (5 mM) | Rat liver GST mix (Sigma) | $2.2 \times 10^{-3}$ | [This work] |
| EDB (37 mM)† | Rat liver GST mix | $2.6 \times 10^{-3}$ | [9: Fig. 1] |
| Solvent | 3–4 (Rat liver) | $2.8 \times 10^{-3}$ | [This work] |
| CDNB (1.2 mM) | 3–4 (Rat liver) | $1.7 \times 10^{-3}$ | [This work] |
| CDNB (1.2 mM) | 3–4 (Rat liver) | $2.5 \times 10^{-3}$ | [This work] |
| EDB (37 mM) | 3–3 (Rat liver) | $2.0 \times 10^{-3}$ | [9] |
| CDNB (1.2 mM) | Human acidic lung GST | $5.3 \times 10^{-3}$ | [11] |

CDNB: 1-Chloro-2,4-dinitrobenzene; EDB: ethylene dibromide; CTX: cefataxin; MP-8: microperoxidase-8; solvent: phosphate buffer pH 6.5, $\mu = 0.10$. Except where noted otherwise $T = 25°$. Standard deviations on $k_{obs}$ are of the order of ±15%.

* Under argon atmosphere.

† Unchanged when repeated by ourselves under argon atmosphere.

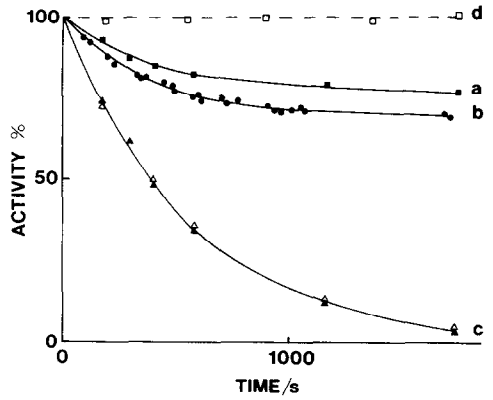


Fig. 1: Typical kinetic curves for the inactivation of GST $\pi$. (a) (—■—) Dilutional inactivation observed on dilution of stock enzyme solution to $1 \times 10^{-7}$ mol/dm$^3$ in buffer. (b) (—●—) Facilitated inactivation on incubation of the enzyme ($1 \times 10^{-7}$ mol/dm$^3$) with the heme-peptide microperoxidase-8 ($4 \times 10^{-7}$ mol/dm$^3$). The data points are the pooled results of three separate experimental runs and are corrected for the dilutational inactivation as shown in kinetic trace (a) by simultaneously running "blanks" in parallel. (c) (—▲— and —△—) Inactivation of GST-$\pi$ on incubation with CDNB ($1 \times 10^{-3}$ mol/dm$^3$). Results from two kinetic runs are shown.

The pseudo first-order rate constants evaluated and used to calculate the solid lines are $2.7 \times 10^{-3}$/sec, $2.2 \times 10^{-3}$/sec and $1.7 \times 10^{-3}$/sec, respectively. (d) (—□—) Protection against inactivation afforded by preincubation of the concentrated enzyme with glutathione (1 mM) for 5 min at 25° followed by dilution into buffer 1 mM in GSH. The enzyme activity was then assayed by adding CDNB alone.

were flushed with argon prior to storage at 4°, and again, subsequent to checking of pH immediately prior to use.

The activity of the enzyme was assayed using the method of Habig and Jakoby [8], the only difference to their methodology being that here DMSO was used for dissolution of the CDNB in place of ethanol (this eliminated ethanol mediated inactivation of the enzyme); the final DMSO concentration was 2% (v/v) in all experiments.

The kinetics of enzyme inactivation were followed by addition of stock concentrated enzyme solution to buffer in the absence or presence of added MP-8, CDNB or EDB. MP-8-facilitated and 'blank'-inactivation experiments were conducted under an argon atmosphere. At fixed times after initiation of incubation the activity was assayed by adding GSH and CDNB simultaneously; or in the case where incubation was carried out in the presence of CDNB, GSH alone. The $Abs_{340\,nm}$ time data was in all cases corrected for the slow spontaneous reaction which occurs between GSH and CDNB.

Pseudo first-order inactivation rate constants ($k$) were obtained by non-linear least squares fitting of the activity/time data to Eqn (1).

$$\text{Activity} = a + be^{-kt} \quad (1)$$

using an iterative Gauss Newton procedure (Program Package "Enzfitter"; Elsevier, 1987).

Values of the inactivation rate constants quoted for literature data were obtained by measurement of data points from enlarged copies of inactivation/time plots, [2, 3, 9–11; Fig. nos as noted in Table 1] followed by non-linear least squares fitting to Eqn (1) as detailed.

*Results and discussion*

In Fig. 1 we show activity/time curves for dilutional MP-8-mediated and CDNB-mediated inactivation of human placental GST $\pi$. Included in the figure are data which illustrate the protection against inactivation afforded by preincubation of the concentrated enzyme with GSH. In Table 1 we summarize results obtained in the present study, and literature data on the inactivation rate constant at pH 6.5 for a range of GST isoenzymes and inactivating agents, both co-substrates and non-substrate ligands. The remarkable invariance of the observed inactivation rate constant, combined with the fact (Fig. 1) that co-substrate/non-substrate ligands affect the extent, but not the rate constant of inactivation, suggests that at pH 6.5 these substances are not inactivating agents *per se*. They merely facilitate inactivation by the same process as that which occurs on dilution of the enzyme in buffer alone. We suggest that this process involves solvation of the hydrophobic catalytic centre of the enzyme, added co-substrate/non-substrate ligands facilitating the extent to which water molecules can penetrate the catalytic centre, thus affecting the extent but not the rate constant for solvational inactivation. We further suggest that protection of the GST from this type of inactivation by pre-incubation with glutathione (see Fig. 1) is a result of the tripeptide sterically hindering the approach of water molecules to the catalytic centre via a polar route.

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*MRC Biomembrane Research Unit
Department of Chemical Pathology
and
†Department of Medical Biochemistry
University of Cape Town
Medical School
Observatory 7925
Republic of South Africa

PAUL A. ADAMS*
RICHARD D. GOOLD†
CYNTHIA N. T. SIKAKANA†

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# Hydrolysis of phthalate esters by purified rat and human liver carboxylesterases

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Because of their wide use as plasticizers and production in millions of tons per year, phthalate esters have become environmental pollutants. Phthalates or metabolites of them are suspected to be hepatocarcinogenic [1, 2] and teratogenic [3] after chronic exposure and/or high dosage. Their metabolism in rats and humans involves the hydrolysis of one ester bond, and further metabolites are formed from the monoesters [1, 4]. Hydrolytic activity has been detected in rat pancreas, liver, mucosa, kidney and lung [5], but apart from the characterization of a pancreatic lipase [6] the identification of mammalian esterases or lipases involved in the metabolism of phthalate esters is unsatisfactory so far. Since in mammals hepatic carboxylesterases are mainly responsible [7, 8] for the hydrolysis of many ester- or amide-type drugs, we determined the action of purified carboxylesterases from rat and human liver on various phthalate diesters, in order to evaluate the contribution of these detoxication enzymes on the metabolism of these xenobiotics.

## *Materials and methods*

*Materials*. Phthalate esters were purchased in highest obtainable purity from Fluka, or Merck (Darmstadt, F.R.G.) (dibutyl, diallyl, dicyclohexyl), except di(2-methoxyethyl), di(2-ethylhexyl) and diisobutyl phthalates which were generous gifts from Badische Anilin- und Sodafabrik, Ludwigshafen, F.R.G. The monobutyl ester was synthesized from equimolar amounts of phthalic acid anhydride and *n*-butanol in the presence of pyridine (2 hr, 95°). After acidification with hydrochloric acid, the monoester was purified by repeated crystallization from ethanol (m.p. of the product 74°).

*Enzymes*. Carboxylesterases were highly purified from rat [9] and human [10] liver microsomes as described earlier.

*Enzyme assays*. The hydrolysis of phenyl butyrate, methyl butyrate, 4-nitrophenyl acetate and palmitoyl-CoA was followed as described previously [11]. Phthalate esters were emulsified by ultrasonication (2 min, 150 W; Branson B 12 sonifier from Branson, Danbury, CT) immediately before assay in 0.1% Triton X-100 to yield 5 mM emulsions (or solutions). After registration of the blank with 5 ml of these substrate emulsions, the purified esterases in 100 μl 10 mM Tris–HCl buffer, pH 8.0, were added and the enzymatic hydrolysis was monitored at pH 8.0 and 37° by the pH-stat method using the autotitrator TTT 11 (Radiometer, Copenhagen, Denmark) with an automatic burette filled with 40 mM NaOH. One unit is defined as the enzymatic generation of one μmole carboxyl-groups per min. Protein was determined by the biuret procedure [9]. The possible enzymatic generation of phthalic acid was further investigated in some of the assays by spectrophotometric determination of the acid according to Takeuchi *et al.* [12].

## *Results and discussion*

Three genetically distinct carboxylesterases (see Refs. 13 and 14 for classification and substrates), namely esterase pI 5.6 (ES-3, acetanilid hydrolase, lysophospholipase), esterase pI 6.0 (ES-8/ED-10, esterase E1, medium-chain monoacylglycerol lipase) and esterase pI 6.2/6.4 (ES-4, long-chain monoacylglycerol lipase) have been identified in rat liver endoplasmic reticulum as the most important xenobiotic-hydrolyzing carboxylesterases of this tissue. From human liver only one prominent detoxifying carboxylesterase is known [10, 15]. With common esterase substrates we measured for the highly purified esterases used in this study the following specific activities: with 1 mM phenyl butyrate at pH 8.0 and 37° rat esterase pI 5.6 192 U/mg, rat esterase pI 6.0 502 U/mg and rat esterase pI 6.2/6.4 140 U/mg; human esterase: 320 U/mg with 10 mM methyl butyrate at pH 8.0 and 30°, 101 U/mg with 0.5 mM 4-nitrophenyl acetate at pH 8.0 and 30°. These values are comparable or higher to activities reported earlier [10, 11].

The activities of these esterases on phthalate esters are summarized in Table 1. The diethyl, diallyl and dibutyl phthalates were generally the best esterase substrates, whereas strong hydrophobic diesters like diethylhexyl phthalate were not significantly hydrolyzed. Incorporation of other detergents as Triton X-100 in the assay (bovine serum albumin, taurocholate, no detergent) did not significantly moderate the activities of human or rat esterase pI 6.2/6.4 (determined with dibutyl phthalate as substrate). Monophthalate esters were not measurably hydrolyzed, and no free phthalic acid could be detected by the photometric method of Takeuchi *et al.* [12] even after prolonged incubation times (120 min, assays only with human esterase). It is therefore concluded that the liver esterases cleave only one ester bond from phthalate diesters. This is in accordance with the observation that charged esters like the monoesters are bad substrates for liver carboxylesterases [11, 15].

When the activities of the three genetically distinct rat liver esterases are compared to each other, the isoenzyme