Mitochondrial aspartate aminotransferase catalyses cysteine S-conjugate β-lyase reactions

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Rat liver mitochondrial aspartate aminotransferase (a homodimer) was shown to catalyse a β-lyase reaction with three nephrotoxic halogenated cysteine S-conjugates [*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine], and less effectively so with a non-toxic cysteine S-conjugate [benzothiazolyl-L-cysteine]. Transamination competes with the β-lyase reaction, but is not favourable. The ratio of β elimination to transamination in the presence of *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine and 2-oxoglutarate is > 100. Syncatalytic inactivation by the halogenated cysteine S-conjugates is also observed. The enzyme turns over approx. 2700 molecules of halogenated cysteine S-conjugate on average for every monomer inactivated. Kidney mitochondria are known to be especially sensitive to toxic halogenated cysteine S-conjugates. Evidence is presented that 15–20 % of the cysteine S-conjugate β-lyase activity towards *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine in crude kidney mitochondrial homogenates is due to mitochondrial aspartate aminotransferase. The possible involvement of mitochondrial aspartate aminotransferase in the toxicity of halogenated cysteine S-conjugates is also discussed.

Key words: halogenated alkenes, mitochondrial toxicity, *S*-(1,2-dichlorovinyl)-L-cysteine, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, thioacylating fragments.

INTRODUCTION

Many halogenated xenobiotics are detoxified through the mercapturate pathway [xenobiotic → glutathione S-conjugate → cysteinylglycine S-conjugate → cysteine S-conjugate ⇆ *N*-acetyl-L-cysteine S-conjugate (mercapturate) → excretion]. However, this pathway can sometimes result in toxification (bioactivation), so that the corresponding halogenated glutathione S-conjugate, cysteine S-conjugates and mercapturate are nephrotoxic. Toxicity results from the action of cysteine S-conjugate β-lyases. These enzymes convert many halogenated cysteine S-conjugates with good leaving groups in the β position into aminoacrylate and a sulphur-containing fragment (RSH). Aminoacrylate is unstable, and is hydrolysed non-enzymically to pyruvate and ammonia. The net reaction (eqn 1) is:

$$RSCH_2CH(CO_2^-)NH_3^+ + H_2O \rightarrow CH_3C(O)CO_2^- + NH_4^+ + RSH \quad (1)$$

Generally, when RSH is stable, the parent cysteine S-conjugate, e.g. benzothiazolyl-L-cysteine (BTC), is not particularly toxic. Toxicity results when the cysteine S-conjugate [e.g., *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) or *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFC)] gives rise to a sulphur-containing fragment that is a reactive, thioacylating agent. Protein lysine residues are particularly susceptible to thioacylation. Although other organs/tissues can be damaged, the proximal tubular regions of the kidney are especially vulnerable to a diverse range of halogenated cysteine S-conjugates (for reviews, see, for example, [1–5]).

Toxic cysteine S-conjugates appear to preferentially target mitochondria (for a recent review, see [6]). For example, when TFEC is administered to rats, a thioacylating fragment labels several kidney mitochondrial (but not cytosolic) proteins [7]. Labelled mitochondrial proteins include HSP60, HSP70, mitochondrial aspartate aminotransferase (mitAspAT), lipoamide succinyltransferase [also known as E2o (equivalent to E2k, where 'o' and 'k' refer to 'oxo' or 'keto' respectively)], dihydrolipoamide dehydrogenase (E3) and aconitase [7–9]. TFEC administration to rats results in a time-dependent loss of kidney 2-oxoglutarate dehydrogenase complex (OGDHC) activity [8] and aconitase activity [9]. Exposure of cultured PC12 cells to TFEC results in pronounced loss of OGDHC and mitAspAT, but not of cytosolic aspartate aminotransferase (cytAspAT), activities, and alterations to the pyruvate dehydrogenase regulatory machinery [10]. OGDHC is also inactivated in TAMH cells exposed to TFEC [9].

At least nine pyridoxal 5′-phosphate (PLP)-dependent enzymes are known to catalyse cysteine S-conjugate β-lyase reactions [6]. Because many of these enzymes are widespread, kidney mitochondria should not be inherently any more susceptible to damage than mitochondria from other organs. Indeed, tricarboxylic acid cycle activity is impaired by halogenated, cysteine S-conjugates in isolated liver mitochondria (e.g. [11]) and, as noted above, in cultured neuron-like PC12 cells [10]. What then accounts for the selective vulnerability of the kidney proximal tubules to toxic halogenated glutathione- and cysteine-S-conjugates *in vivo*? From the available evidence, damage appears to stem from the enterohepatic circulation of the glutathione-, L-cysteine- and *N*-acetyl L-cysteine (mercapturate) S-conjugates. Glutathione S-conjugates accumulate mainly in the kidney, and are hydrolysed into component amino acids

Abbreviations used: AlaAT, alanine aminotransferase; BTC, benzothiazolyl-L-cysteine; CTFC, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; cytAspAT, cytosolic aspartate aminotransferase; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; mitAspAT, mitochondrial aspartate aminotransferase; OGDHC, 2-oxoglutarate dehydrogenase complex; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

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(including cysteine S-conjugate) by the action of γ-glutamyl transferase and dipeptidase(s) on the renal brush-border membranes (e.g. [12,13]). The cysteine S-conjugate, which is formed in the tubular lumen, is reabsorbed and transferred to the liver, acetylated to form the mercapturate and excreted in the urine [12,13]. Other factors contributing to renal proximal tubule damage include an avid uptake system for mercapturates in kidney tubules [14] and the presence of acylases that deacylate mercapturates in the kidney [15]. (For a discussion of the enterohepatic circulation and metabolism of S-conjugates, see [3].)

In most reports, doses of halogenated, cysteine S-conjugates that induce renal damage *in vivo* are not detectably hepatotoxic (e.g. [16]), or are only slightly so [17]. Thus glucose metabolism and urea synthesis in liver are not expected to be directly perturbed by nephrotoxic doses of most halogenated cysteine S-conjugates. However, because urea is excreted by the kidneys and glucose is reabsorbed by the kidneys, cysteine S-conjugate-induced damage to the kidney is associated with increased plasma urea/blood urea nitrogen and increased glucose excretion in the urine (e.g. [8,17,18]).

Halogenated xenobiotics that are present in the workplace/environment, and that are metabolized, at least in part, to toxic cysteine S-conjugates, include tetrafluoroethylene, trichloroethylene and tetrachloroethylene. The corresponding toxic cysteine S-conjugates are TFEC, DCVC and *S*-(1,2,2-trichlorovinyl)-L-cysteine respectively. Considerable effort has been devoted to identifying mammalian cysteine S-conjugate β-lyases that might contribute to this bioactivation process. Most cysteine S-conjugate β-lyases identified to date [kynureninase, cytAspAT, alanine aminotransferase (AlaAT), cytosolic- and mitochondrial-branched-chain aminotransferases], but not cytosolic glutamine transaminase K, are syncatalytically inactivated [6]. In order to understand better the mechanism by which halogenated cysteine S-conjugates are toxic to kidney mitochondria, it is important to characterize all the potential cysteine S-conjugate β-lyases present in kidney mitochondria. Note that, because of the unique enterohepatic circulation of S-conjugates, the mitochondrial PLP-dependent enzymes responsible for selective damage to kidney by halogenated cysteine S-conjugates are not necessarily unique to that organ.

Indirect evidence in the literature suggests that the widely distributed mitAspAT might catalyse a cysteine S-conjugate β-lyase reaction. For example, as noted above, kidney mitAspAT is adducted by a thioacylating fragment after rats are administered TFEC [7]. In this case, adduction may be due to a cysteine S-conjugate β-lyase reaction catalysed by the enzyme itself. The inactivation of mitAspAT by TFEC in PC12 cells [10] and in rat tissue homogenates [18] provides further indirect evidence that cysteine S-conjugates are processed at the active site of the enzyme. We now show directly that highly purified rat liver mitAspAT possesses cysteine S-conjugate β-lyase activity, and that processing of cysteine S-conjugates at the active site leads to inactivation. The present results suggest that halogenated cysteine S-conjugates may be toxic to kidney mitochondria, in part through processing at the active site of mitAspAT, and in part by destruction of the normal transaminase activity.

EXPERIMENTAL

Reagents and enzymes

Ammediol (2-amino-2-methyl-1,3-propanediol), sodium thiosulphate, Tris, Hepes, β-chloro-L-alanine/HCl, EDTA, 2,4-dinitrophenylhydrazine, NADH, NAD^+, ADP, rabbit muscle lactate dehydrogenase [LDH, type XXXIX; 720 units/mg of protein in 50 % (v/v) glycerol; 2.9 mg/ml], beef liver glutamate dehydrogenase [GDH, type II; 50 units/mg of protein in 50 % glycerol; 10 mg/ml], pig heart malate dehydrogenase (MDH; 910 units/mg in 50 % glycerol, 50 mM potassium phosphate buffer, pH 7.5; 5.6 mg/ml], pig heart cytAspAT (type I; 270 units/mg suspension in 3 M ammonium sulphate, 0.05 M maleate and 2.5 mM 2-oxoglutarate), pig heart AlaAT (120 units/mg suspension in 1.8 M ammonium sulphate), 30 % (v/v) hydrogen peroxide, the monosodium salts of aspartate and glutamate, and the sodium salts of pyruvate and 2-oxoglutarate were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). The specific activities of the commercial enzymes were determined to be close to those stated by the supplier. Rat-liver mitAspAT [1.35 mg/ml in 20 mM Tris/HCl buffer, pH 8.3, containing 0.1 mM EDTA, < 50 mM NaCl and 0.2 % (w/v) sodium azide; 410 units/mg at 37 °C (see below)] was expressed in *Escherichia coli* and purified, as described previously [19]. Protein concentrations were estimated spectrophotometrically from the absorbance at 280 nm using a molar absorption coefficient of 62500 $M^{-1} \cdot cm^{-1}$ and a M_r of the monomer of 44600 [20]. SDS/PAGE and mass spectral analysis showed the enzyme to be > 99 % homogeneous; no contaminating bacterial proteins were detected (results not shown). TFEC [21] and BTC (acetate salt) [22] were synthesized as described previously. DCVC and CTFC were generously given by Dr James L. Stevens (Lilly Research Laboratories, Greenfield, IN, U.S.A.). Stock solutions of β-lyase substrates used in the present experiments, namely: 20 mM BTC in 100 mM ammediol-HCl buffer, pH 9.0; 40 mM TFEC in 100 mM Tris/HCl buffer, pH 8.5; 40 mM CTFC in 100 mM Tris/HCl buffer, pH 8.5; 20 mM DCVC in 100 mM Tris/HCl buffer, pH 8.5; and 100 mM β-chloro-L-alanine/HCl (neutralized with NaOH) in distilled water, were stored at −20 °C.

Enzyme measurements

The mitAspAT assay was a modification of the MDH-coupled assay published previously [19] for multi-well plate analyses. The reaction mixture (0.2 ml) contained 100 mM Hepes, 10 mM aspartate, 6 mM 2-oxoglutarate, 0.1 mM NADH and 0.6 μg of MDH (the pH of the assay solution was adjusted to 7.5 with NaOH). The well plate was pre-warmed to 37 °C and the reaction was initiated by addition of a solution containing mitAspAT (1–10 μl). The disappearance of absorbance of NADH at 340 nm (ε 6230 $M^{-1} \cdot cm^{-1}$) was continuously monitored in a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). The blank was a reaction mixture containing no added enzyme. In controls for experiments showing inactivation by the various β-lyase substrates, no appreciable loss of activity (< 10 %) was noted when the enzyme (10–100 μg/ml) was incubated for 10 h at 37 °C in 100 mM Hepes/NaOH buffer, pH 7.5. GDH, LDH, cytAspAT and mitAspAT in crude cytosolic and mitochondrial kidney fractions were assayed as described previously [10]. A unit of enzyme activity for all enzymes investigated here is the amount that catalyses the formation of 1 μmol of product/min at 37 °C.

Measurement of pyruvate, ammonia and glutamate

In most cases, pyruvate was measured as its 2,4-dinitrophenylhydrazone. To a 20 μl solution containing pyruvate was added 20 μl of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After incubation at 37 °C for 10 min, 160 μl of 1 M NaOH was added, and the absorbance at 430 nm was determined (ε 15000 $M^{-1} \cdot cm^{-1}$) within 2 min in a SpectraMax 96-well plate analyser against a blank consisting of 20 μl of water, carried

through the same procedure. In some experiments, 0.5 mM 2-oxoglutarate was present in the assay mixture and this resulted in a slightly elevated blank absorbance. However, the presence of 0.5 mM 2-oxoglutarate had no effect on the slope of the standard curve for pyruvate. In some cases, where the concentration of 2-oxoglutarate in the assay mixture was higher, pyruvate was measured with LDH. In this procedure, 180 μl of a mixture containing 0.1 mM NADH, 7.5 μg of LDH and 100 mM potassium phosphate buffer, pH 7.4, was added to a 20 μl solution containing pyruvate. The decrease in absorbance at 340 nm was monitored in a SpectraMax96-well plate analyser at 37 °C. The conversion of lactate into pyruvate was complete within 2 min.

Ammonia was measured enzymically with GDH. To a 20 μl solution containing ammonia was added 180 μl of a mixture containing 0.1 mM NADH, 50 μg of GDH, 0.1 mM ADP, 25 mM 2-oxoglutarate and 100 mM potassium phosphate buffer, pH 7.4. The decrease in absorbance at 340 nm was monitored. The conversion of ammonia to L-glutamate was complete in 10 min at 37 °C, at which time the slow drift in absorbance loss at 340 nm in the sample well equalled that in the blank well.

L-Glutamate was measured enzymically with GDH by the procedure of Lowry and Passonneau [23], as modified for multi-well plate analyses. To a 20 μl solution containing glutamate and a low amount of mitAspAT (3.4 μg) was added 180 μl of a reaction mixture containing 2 mM NAD^+, 50 mM Tris/acetate buffer, pH 8.5, 0.1 mM ADP, 10 mM hydrogen peroxide and 2 μg of GDH. The increase in absorption at 340 nm at 37 °C due to reduction of NAD^+ was complete within 30 min. A high background occurred when relatively large amounts of mitAspAT (34 μg) were present. In that case, the 20 μl sample was incubated with 20 μl of 10 mM hydrogen peroxide for 1 h at 37 °C prior to addition of ADP, NAD^+ and GDH. The interfering background absorbance was destroyed by the pre-treatment with hydrogen peroxide.

Fractionation of rat kidney homogenates

Crude cytosolic and mitochondrial fractions were prepared from kidneys of male 3-month-old Fischer 344 x Brown Norway F_1 rats. The rats were killed and the kidneys were removed, weighed and homogenized in 2 vols. of ice-cold 0.25 M sucrose by means of a glass-to-glass hand-held homogenizer. The crude mitochondrial pellet was prepared by centrifugation at 10000 ***g*** for 15 min, and at 4 °C. The mitochondrial pellet and the supernatant (approx. 2.8 ml/g wet weight of kidney) were stored separately at −80 °C. [Freezing at −80 °C does not result in any loss of cysteine S-conjugate β-lyase activity (using TFEC as substrate) in either fraction.] On thawing, the mitochondrial pellet was suspended in a volume comparable with the cytosolic fraction of ice-cold 10 mM potassium phosphate buffer, pH 7.4 (approx. 2.8 ml/g wet weight of kidney). The enzyme activities in the crude cytosolic plus mitochondrial fractions were assumed to account for 100 % of the total.

Statistics

For determinations where n is $\geqslant 3$, the means ± S.E.M. are reported. Statistical comparisons were performed using the Mann–Whitney U test; $P \leqslant 0.05$ was considered to be significant.

RESULTS

Rat liver mitAspAT has cysteine S-conjugate β-lyase activity

Table 1 shows that the enzyme exhibits β-lyase activity towards a number of cysteine S-conjugates. Activity is greater with the toxic cysteine S-conjugates (TFEC, CTFC and DCVC) than with non-toxic BTC. In preliminary studies, it was found that maximal pyruvate formation requires the simultaneous presence of 2-oxoglutarate (results not shown). In the experiment shown in Table 1, 0.5 mM 2-oxoglutarate was present. This level of 2-oxoglutarate ensures that the blank reading is not unacceptably high for measurement of pyruvate as its 2,4-dinitrophenylhydrazone. At the same time, this concentration is similar to the dissociation constants for binding of 2-oxoglutarate to various AspATs [24,25]. For comparison, β-chloro-L-alanine was also tested as a β-lyase substrate of rat liver mitAspAT (Table 1). Although the enzyme exhibits a higher V_{max} with β-chloro-L-alanine than with the cysteine S-conjugates, the K_m value is higher for β-chloro-L-alanine. As a result, the relative V_{max}/K_m ratio for β-chloro-L-alanine is comparable with that of the cysteine S-conjugates, and in one case (with DCVC) is considerably lower (Table 1).

Table 1 Rat liver mitAspAT-catalysed β-lyase reactions with cysteine S-conjugates and β-chloro-L-alanine

For experiments with TFEC, CTFC, DCVC and BTC, the reaction mixture (20 μl) contained cysteine S-conjugate, 200 mM Hepes/NaOH buffer, pH 7.5, 0.5 mM 2-oxoglutarate and 2.7 μg of mitAspAT. After incubation for 3–10 min at 37 °C, the amount of pyruvate formed was determined by the 2,4-dinitrophenylhydrazone procedure. At least six triplicate concentrations of substrate were used for each kinetic determination. K_m and V_{max} values were obtained from standard Lineweaver–Burk plots. Because the enzyme is eventually inactivated by the cysteine S-conjugates, times of incubation were chosen from pilot experiments so that no more than 5% inactivation of the enzyme had occurred at the end of the incubation. For experiments with β-chloro-L-alanine, the reaction mixture was similar to that used for the cysteine S-conjugates, except that α-oxoglutarate was not included in the assay mixture, incubation was for 0.5 min, and 6.75 μg of enzyme was used. In a separate experiment, it was shown that the loss of enzyme activity at 0.5 min at the maximal concentration of β-chloro-L-alanine (200 mM) employed in the kinetic experiments was approx. 10%.

Substrate	Apparent K_m (mM)	V_{max} (nmol/min per μg)	Relative V_{max}/K_m
TFEC	25	2	1
CTFC	40	5.5	1.7
DCVC	2.5	3	15
BTC	2.8	0.18	0.8
β-Chloro-L-alanine	120	28	2.9

Stoichiometry of the β-lyase reaction

Rat liver mitAspAT was incubated for 2 h in the presence of 5 mM 2-oxoglutarate and either 20 mM TFEC or 10 mM DCVC, at which time the enzyme was $> 95\,\%$ inactivated (see below). Aliquots of the reaction mixture were then analysed for ammonia, pyruvate and L-glutamate (Table 2). Note that a higher level of 2-oxoglutarate was included in this experiment (5 mM) compared with that in the previous experiment (0.5 mM). It was reasoned that, if appreciable transamination competed with the β-lyase reaction, more glutamate would be generated in the presence of 5 mM 2-oxoglutarate and hence the accuracy of the glutamate determination would be greater. For both DCVC and TFEC, the amount of pyruvate formed was stoichiometric with that of ammonia. However, L-glutamate could not be detected in this experiment ($< 0.5\,\%$ that of pyruvate formation). In a separate experiment, it was found that stoichiometric amounts of pyruvate and ammonia were formed also from β-chloro-L-alanine in the presence of mitAspAT (Table 2). In the case of β-chloro-L-alanine, however, inclusion of 2-oxoglutarate in the reaction mixture was not necessary to ensure maximal β-lyase activity (see below).

Table 2 Rat liver mitAspAT-catalysed formation of pyruvate and ammonia from β-lyase substrates

For experiments with TFEC and DCVC, the reaction mixture (100 μl) contained 200 mM Hepes/NaOH buffer, pH 7.5, cysteine S-conjugate, 5 mM 2-oxoglutarate and 6.75 μg of mitAspAT. After incubation at 37 °C for 2 h, aliquots (10–20 μl) were withdrawn and assayed separately for pyruvate (with the LDH procedure), ammonia and L-glutamate ($n = 3$). The product is the amount formed per 100 μl of reaction mixture. For experiments with β-chloro-L-alanine, the reaction mixture (50 μl) contained β-chloro-L-alanine, 100 mM Hepes/NaOH buffer, pH 7.5. and 6.75 μg of mitAspAT. After incubation for 2 min at 37 °C, the reaction was terminated by immersing the tube in a boiling-water bath. Aliquots (20 μl) were then removed for analysis of pyruvate (by the 2,4-dinitrophenylhydrazone procedure) and ammonia (by the GDH procedure; $n = 3$). The product is the amount formed per 50 μl reaction mixture. –, not done.

Substrate	Concentration (mM)	Product (nmol)		
		Pyruvate	Ammonia	L-Glutamate
TFEC	20	402 ± 25	410 ± 32	< 2
DCVC	10	422 ± 22	400 ± 38	< 2
β-Chloro-L-alanine	25	39.3 ± 0.3	43.3 ± 7.3	–
	50	71.0 ± 1.3	75.5 ± 8.3	–

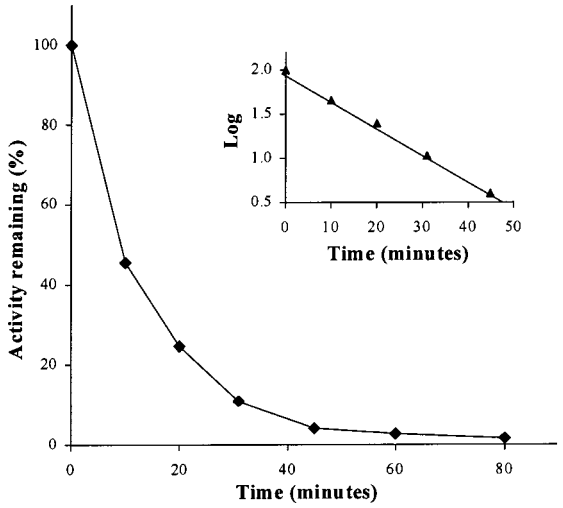

Figure 2 Inactivation of rat liver mitAspAT by β-chloro-L-alanine

The reaction mixture (0.1 ml) containing 100 mM Hepes/NaOH buffer, pH 7.5, 25 mM β-chloro-L-alanine and 1.35 μg of mitAspAT was incubated at 37 °C in a small snap-top tube. At intervals, 2 μl aliquots were withdrawn and the inactivation process was quenched by addition of the aliquot to a standard reaction mixture lacking 2-oxoglutarate in a well plate maintained at 4 °C. After removing the last 2 μl aliquot at 80 min, the plate was warmed to 37 °C and the activity remaining in all aliquots was simultaneously determined by addition of 2-oxoglutarate to each well. The inset shows a plot of $\log_{10}$ remaining activity against time. No loss of enzyme activity occurred in the control, in which mitAspAT was incubated in buffer in the absence of β-chloro-L-alanine.

Inactivation of rat liver mitAspAT by TFEC

Because several PLP-containing enzymes are inactivated by cysteine S-conjugate β-lyase substrates [6], the possibility was considered that rat liver mitAspAT might also be inactivated by these substrates. This was found to be the case. The progress curve for inactivation of enzyme by TFEC is biphasic (Figure 1). After approx. 1 h, the relative rate of inactivation is greatly lowered so that, at this point, approx. 35–40 % of the original enzyme is now resistant to inactivation. However, in the presence of 0.5 mM 2-oxoglutarate, no such change is observed at 1 h: inactivation proceeds in a close-to-monophasic manner until at least 98 % of the enzyme is inactivated ($t_{1/2} \approx 30$ min), and the extent of inactivation is greater at all time points (Figure 1).

The biphasic nature of the TFEC-induced inactivation in the absence of added 2-oxo acid substrate is presumably due to transamination of PLP cofactor to pyridoxamine 5′-phosphate (PMP) cofactor competing with β-elimination at the active site (cf. [26]; also see the discussion). As noted above, however, initial attempts failed to demonstrate transamination between TFEC and 5 mM 2-oxoglutarate directly (Table 2). The experiment shown in Table 2 was repeated, except that 10-fold more enzyme (34 μg in a 50 μl reaction mixture) was present. After 2 h incubation at 37 °C, 20 μl of the reaction mixture was analysed for L-glutamate, as described in the Experimental section. The

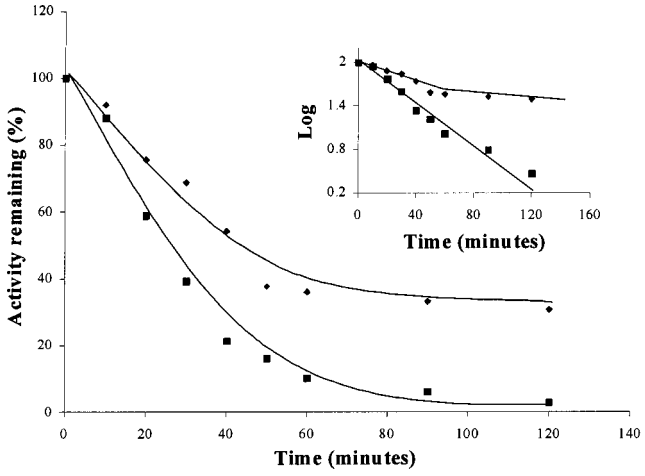

Figure 1 Inactivation of rat liver mitAspAT by TFEC

The reaction mixture (50 μl) containing 200 mM Hepes/NaOH buffer, pH 7.5, 10 mM TFEC and 0.27 μg of mitAspAT was incubated at 37 °C. At intervals, aliquots were withdrawn and assayed for remaining enzyme activity. The curves represent inactivation in the absence of 2-oxoglutarate (◆) and inactivation in the presence of 0.5 mM 2-oxoglutarate (■). The inset shows a plot of $\log_{10}$ remaining activity against time. In a separate experiment, it was shown that the enzyme is stable for at least 3 hours in a reaction mixture containing 0.5 mM 2-oxoglutarate (results not shown).

Table 3 Ratio of turnover to inactivation for various β-lyase substrates

The reaction mixture (20 μl) contained 200 mM Hepes/NaOH buffer, pH 7.5, β-lyase substrate, 0.5 mM 2-oxoglutarate and 0.27 μg of rat liver mitAspAT. After 3 h incubation at 37 °C in a small snap-top tube, the amount of pyruvate formed was determined by the 2,4-dinitrophenylhydrazone method. In a separate experiment, it was shown that this treatment is sufficient to completely inactivate the enzyme for all β-lyase substrates shown. The ratio is nmol of pyruvate formed per monomer of enzyme inactivated.

Substrate	Concentration (mM)	Ratio	*n*
TFEC*	20	2700 ± 125	3
CTFC*	20	2720 ± 60	3
DCVC*	10	2750 ± 90	3
β-Chloro-L-alanine	50	3850 ± 50	9

* Different from the value obtained with β-chloro-L-alanine ($P = 0.005$).

level of L-glutamate detected (approx. 5 nmol) was hardly above the noise of the background, yielding a maximal turnover of 25 nmol/1.55 nmol of enzyme monomer, or approx. 16 transamination events on average before inactivation of each monomer occurs. When these assay mixtures were spiked with 2 μl of 20 mM L-glutamate (40 nmol), the expected increase in absorbance at 340 nm occurred (results not shown), showing that the glutamate detection system was not compromised.

Because chloride is a better leaving group than RS^-, the ratio of β-elimination to transamination should be more favourable for β-chloro-L-alanine than for the cysteine S-conjugates, and this was found to be the case. In the presence of 25–50 mM β-chloro-L-alanine, the relative rate of inactivation of rat liver mitAspAT is independent of enzyme concentration (results not shown), and a build-up of enzyme resistant to syncatalytic inactivation (i.e. the PMP form) is insignificant (Figure 2). These findings suggest that the ratio of transamination to β-elimination is negligible in the case of β-chloro-L-alanine.

Ratio of turnover to inactivation in the presence of various β-lyase substrates

Table 3 shows the amount of pyruvate generated per enzyme monomer inactivated by various β-lyase substrates. The amount of pyruvate generated was similar for the three toxic halogenated cysteine S-conjugates (TFEC, CTFC and DCVC; approx. 2700 nmol/nmol of enzyme subunit inactivated), but significantly less ($P = 0.005$) than that observed for β-chloro-L-alanine (3850 nmol/nmol of enzyme subunit inactivated). Because a maximum of 16 nmol of glutamate could be detected per nmol of subunit inactivated for the toxic cysteine S-conjugates, these results suggest that for these β-lyase substrates the ratio of elimination to transamination at the active site is >100 (i.e. 2700:16).

Effect of thiosulphate on the inactivation of rat liver mitAspAT by β-chloro-L-alanine and TFEC

Cavallini et al. [27] showed that thiosulphate partially protects pig heart cytAspAT against inactivation by serine *O*-sulphate and by cysteine sulphinic acid (β-lyase substrates). Evidence was presented that the protection is due to trapping of aminoacrylate intermediate as cysteine *S*-sulphonate [27] (eqn 2):

$$CH_2 = C(NH_2)CO_2H + HSSO_3H \rightarrow HO_3SSCH_2CH(NH_2)CO_2H \quad (2)$$

In the present work, thiosulphate was shown to protect against inactivation by TFEC, DCVC or β-chloro-L-alanine (Table 4). The protective effect of thiosulphate against inactivation by the halogenated cysteine S-conjugates is, however, less than that observed against inactivation by β-chloro-L-alanine.

Table 4 Effect of thiosulphate on the inactivation of rat liver mitAspAT by various β-lyase substrates

The reaction mixture (50 μl) contained 200 mM Hepes/NaOH buffer, pH 7.5, 0.27 μg of enzyme, β-lyase substrate and, where indicated, 20 mM thiosulphate. The reaction mixtures containing TFEC or DCVC also contained 0.5 mM 2-oxoglutarate. After incubation at 37 °C for 15 min, a 2 μl aliquot was withdrawn and assayed for remaining enzyme activity (aminotransferase assay) for three independent experiments. The control for this series was enzyme incubated for 20 min in 200 mM Hepes buffer. In a separate experiment, it was shown that incubation of 0.27 μg of the enzyme in 50 μl of 200 mM Hepes buffer for 20 min in the presence of (a) 0.5 mM 2-oxoglutarate, (b) 20 mM thiosulphate or (c) 0.5 mM 2-oxoglutarate plus 20 mM thiosulphate had no effect on the aminotransferase activity when 2 μl aliquots were withdrawn and assayed by the standard aminotransferase assay (results not shown).

β-Lyase substrate	Concentration (mM)	Thiosulphate	Activity remaining (%)
Control			[100 ± 3]
β-Chloro-L-alanine	50	−	18.8 ± 0.8
β-Chloro-L-alanine	50	+	74.5 ± 10.4*
TFEC	20	−	39.3 ± 3.3
TFEC	20	+	46.4 ± 4.3*
DCVC	5	−	19.3 ± 2.9
DCVC	5	+	28.3 ± 0.9*

* Significantly different from activity remaining in the corresponding incubation mixtures lacking thiosulphate ($P = 0.05$).

Table 5 Comparison of TFEC β-lyase activity with those of selected marker enzymes in rat kidney cytosol and mitochondria

The TFEC β-lyase reaction mixture (20 μl) contained 20 mM TFEC, 0.5 mM 2-oxoglutarate, 200 mM Hepes/NaOH buffer, pH 7.2, and 2 μl of cytosol or mitochondrial suspension. After incubation at 37 °C for 5–10 min the pyruvate formed was measured by the 2,4-dinitrophenylhydrazone procedure. The blank was 18 μl of assay mixture, to which was added 2 μl of homogenate after addition of the 2,4-dinitrophenylhydrazine reagent. Other enzymes were measured as indicated in the Experimental section, except that the mitochondrial and cytosolic fractions were diluted 10-fold in 10 mM potassium phosphate buffer before assay. Data are the average values for kidneys from three rats.

Enzyme	Activity (μmol/min per gram of wet weight of kidney)	
	Cytosol	Mitochondria
TFEC lyase*	2.92 ± 0.31	0.58 ± 0.03
LDH	13.2 ± 1.1	0.58 ± 0.02
GDH	0.65 ± 0.03	3.4 ± 0.2
cytAspAT	19.9 ± 0.9	1.3 ± 0.2
mitAspAT	4.52 ± 1.52	35.8 ± 1.3

* TFEC lyase represents a composite of several enzyme activities (see the text for further details).

TFEC β-lyase activity in rat kidney subcellular fractions

The cysteine S-conjugate β-lyase (20 mM TFEC as substrate) activities in rat kidney cytosol and mitochondria are shown in Table 5. LDH and cytAspAT are cytosolic markers; GDH and mitAspAT are mitochondrial markers (however, see the Discussion). Therefore, on the basis of relative activities of LDH and cytAspAT in the cytosolic and mitochondrial fractions, only a small percentage of the total cytosolic proteins is in the mitochondrial fraction (4–6%) (Table 5). On the basis of relative

Table 6 Comparison of cysteine S-conjugate β-lyase activities of pig heart cytAspAT, pig heart AlaAT and rat liver mitAspAT

The reaction mixtures contained 20 mM TFEC (or 10 mM BTC), 0.5 mM 2-oxoglutarate, 100 mM Hepes/NaOH buffer, pH 7.4, and 2 μg of cytAspAT (or 14 μg of AlaAT or 2.7 μg of mitAspAT) in a final volume of 20 μl. After incubation at 37 °C for the time indicated, pyruvate formation was measured by the 2,4-dinitrophenylhydrazone procedure ($n = 3$). In preliminary experiments, it was shown that the rate of pyruvate formation is linear for 10 min, except in the case of cytAspAT/BTC (results not shown).

Enzyme	Cysteine S-conjugate	Incubation time (min)	Pyruvate formed (nmol)	Relative rate*
cytAspAT	TFEC	10	3.6 ± 0.6	1
	BTC	30	< 0.5	< 0.1
AlaAT	TFEC	10	8.0 ± 0.4	0.32
	BTC	10	6.6 ± 0.7	0.26
mitAspAT	TFEC	10	27.3 ± 1.3	5.6
	BTC	10	4.7 ± 0.5	0.97

* The data in this column were normalized to initial rate of pyruvate formed/μg of enzyme, or 0.18, 0.057 and 1.01 nmol/min per μg of protein for cytAspAT, AlaAT and mitAspAT respectively.

activities of GDH and mitAspAT in the cytosolic and mitochondrial fractions, only a small percentage of the total mitochondrial proteins is in the cytosolic fraction (11–16 %) (Table 5). The percentage of the total TFEC β-lyase activity in the mitochondrial fraction (17 %) is considerably greater than the percentage of contaminating cytosolic proteins in this fraction (4–6 %). Because the specific activity of TFEC lyase in the cytosolic fraction is 2.92 μmol/min per gram of wet weight, with only a small contribution from mitochondrial enzymes (approx. 0.58×0.16), the data suggest a maximum contamination of the mitochondrial fraction by cytosolic TFEC lyases of 0.175 (i.e. 2.92×0.06) μmol/min per gram of wet weight. Therefore, since the total TFEC lyase activity in the crude mitochondrial fraction is 0.58 μmol/min per gram of wet weight, at least 70 % [(0.58–0.175)/0.58×100], or 0.4 μmol/min per gram of wet weight, of the TFEC β-lyase activity in the mitochondrial fraction must be due solely to mitochondrial enzymes.

Comparison of cysteine S-conjugate β-lyase activities of pig heart cytAspAT and pig heart AlaAT to that of rat liver mitAspAT

Table 6 shows that pig heart cytAspAT and AlaAT can catalyse a cysteine S-conjugate β-lyase reaction with TFEC. In addition, pig heart AlaAT, but not cytAspAT, can catalyse a cysteine S-conjugate β-lyase reaction with BTC.

Table 6 shows that the initial rates of β-lyase activity with 20 mM TFEC are 1.01, 0.18 and 0.057 nmol/min per μg of protein for mitAspAT, cytAspAT and AlaAT respectively. These rates are, respectively, approx. 0.25, 0.1 and 0.05 % the rates of transamination (410, 270 and 120 μmol/min per mg of protein respectively) exhibited with optimal concentrations of 'natural' substrates. Because the three enzymes are homodimers of similar M_r values, the data show that, under the conditions of the assay, rat liver mitAspAT is approx. 6 times more effective as a TFEC β-lyase than is pig heart cytAspAT, and approx. 16 times more effective than is pig heart AlaAT.

DISCUSSION

Our results (Table 6) confirm previous reports that highly purified cytAspAT and AlaAT can catalyse β-lyase reactions with toxic halogenated cysteine S-conjugates [18,28–32]. Our findings show that a highly purified preparation of mitAspAT can also catalyse an effective β-lyase reaction with toxic halogenated cysteine S-conjugates, and that turnover of these compounds leads to slow syncatalytic inactivation. However, the cysteine S-conjugate β-lyase activity of mitAspAT (at least with TFEC as a substrate) is considerably greater than that of cytAspAT and AlaAT (Table 6).

The relatively strong cysteine S-conjugate β-lyase activity of mitAspAT might have important toxicological ramifications. As mentioned in the Introduction, mitochondrial proteins are targeted by a fragment released from TFEC. The present results suggest that mitAspAT has a role in such targeting. On the basis of the mitAspAT activity in the mitochondrial fraction of rat kidneys (i.e. 35.8 μmol/min per gram of kidney wet weight; Table 5) and the ratio of TFEC β-lyase activity (1.01 μmol/min/mg; Table 6) to aspartate/2-oxoglutarate aminotransferase activity (410 μmol/min per mg) of the highly purified rat liver mitAspAT, one can calculate that mitAspAT contributes 0.088 μmol/min per gram of kidney wet weight [i.e. $35.8 \times (1.01/410)$] to the total TFEC β-lyase activity in the rat liver mitochondrial fraction. The total TFEC β-lyase activity of this fraction is 0.58 μmol/min per gram of kidney wet weight (Table 5). Thus the minimum contribution of mitAspAT to the total TFEC β-lyase activity of rat kidney mitochondria is 15 % [i.e. $(0.088/0.58) \times 100$]. However, because up to one-third of the TFEC β-lyase activity of the mitochondrial fraction may be due to contaminating cytosolic proteins (see the Results section), the contribution of mitAspAT to the mitochondrial fraction may be as high as 20 %.

The apparent K_m exhibited by mitAspAT towards TFEC is relatively high (25 mM), but smaller for DCVC (2.5 mM, Table 1). The high K_m suggests that binding of TFEC to mitAspAT *in vivo* might be limited. However, it should be borne in mind that the K_m values exhibited by purified AspAT isoenzymes towards the natural substrate L-glutamate are also quite high (approx. 9–15 mM; [24,25]). Moreover, as mentioned in the Introduction, kidney mitAspAT is labelled *in vivo* by a thioacylating fragment after rats are treated with TFEC [7]. A reasonable explanation for this finding is that the labelling is self-induced. It will be important in future studies to determine the mechanism whereby toxic halogenated cysteine S-conjugates, such as TFEC and DCVC, are accumulated in the mitochondria and come in contact with mitAspAT.

Accumulation of toxic halogenated cysteine S-conjugates in mitochondria and their conversion into a reactive thioacylating fragment by mitAspAT may lead to disruption of energy metabolism in the kidney by at least two mechanisms. We have shown previously that 24 h exposure of PC12 cells to 1 mM TFEC leads to approx. 90 % loss of endogenous mitAspAT activity; cytAspAT is not significantly affected (although it is inactivated by TFEC *in vitro*) [10]. It has not yet been determined whether mitAspAT is inactivated in rat kidney *in vivo* by TFEC, but thioacylation of this protein *in vivo* [7] suggests that it might be. mitAspAT is an important component of the malate/aspartate shuttle for the passage of reducing equivalents across the mitochondrial membrane (cf. [33], and references cited therein). In kidney, this shuttle is thought to control Na^+ reabsorption [34]. Thus disruption of the malate/aspartate shuttle in kidney might lead to serious imbalance in ion regulation. In addition, as noted in the Introduction, the E2 and E3 subunits of rat kidney OGDHC are targeted by TFEC *in vivo* [8], and OGDHC is strongly inactivated in both PC12 and TAMH cells exposed to TFEC [9,10]. The thioacylating fragment released from TFEC is extremely reactive and is unlikely to diffuse far from its site of formation [3,5]. Much evidence suggests

that enzymes of the TCA cycle (including OGDHC) and ancillary enzymes (such as mitAspAT) are arranged in supramolecular complexes (i.e. 'metabolons') (e.g. [35]). It is possible that the close juxtapositioning of mitAspAT to OGDHC facilitates thioacylation and inactivation of OGDHC subunits [6,9].

From a toxicological/pharmacological point of view, it will be important to define the mechanisms whereby mitAspAT is inactivated by toxic halogenated cysteine S-conjugates. It has been known for many years that pig heart AlaAT, cytAspAT and mitAspAT can catalyse a β-lyase reaction with amino acids containing a good leaving group in the β position, such as L-serine *O*-sulphate and β-chloro-L-alanine, and that processing at the active site leads to syncatalytic inactivation (e.g. [26,36,37]). The present work extends these previous studies to show that rat liver mitAspAT also has β-lyase activity towards β-chloro-L-alanine, and is syncatalytically inactivated by this substrate. As was found for the pig heart enzyme (K_m 0.2 M; [37]), rat liver mitAspAT exhibits a high K_m (0.12 M; Table 1) towards β-chloro-L-alanine. The cysteine S-conjugates bind more effectively to mitAspAT than does β-chloro-L-alanine (lower K_m values), but maximal turnover rates with the cysteine S-conjugates are lower (Table 1), presumably because RS^- is not as effective a leaving group as Cl^-. The more effective binding of the cysteine S-conjugates to mitAspAT may be due to a 'preference' for amino acids somewhat larger than β-chloro-L-alanine. In this regard, it is interesting to note that aromatic amino acids, including phenylalanine, tyrosine and tryptophan, are transaminase substrates of mitAspAT from rat liver [38] and pig heart [39].

At first it was assumed that the aminoacrylate generated from the β-lyase reaction inactivates cytAspAT by alkylating an essential active-site residue [36]. This mechanism may hold for inactivation of bacterial D-amino acid aminotransferase by β-chloro-D-alanine [40], but it apparently does not hold for inactivation of glutamate decarboxylase or cytAspAT by L-serine *O*-sulphate [41,42]. Metzler and co-workers have presented strong evidence that aminoacrylate formed from L-serine *O*-sulphate reacts with the PLP cofactor, forming a PLP–pyruvate aldol product that remains attached to the active site, thereby inactivating these enzymes [41,42]. Because thiosulphate partially protects against inactivation of mitAspAT by the cysteine S-conjugates (Table 4), at least part of the inactivation may be due to aminoacrylate addition to an active-site nucleophile. However, two of the present findings suggest that an additional mechanism may operate. First, the protection by thiosulphate is significantly less pronounced against the halogenated cysteine S-conjugates than against β-chloro-L-alanine (Table 4). Secondly, the ratio of pyruvate formed to monomer inactivated is significantly less in the presence of the cysteine S-conjugates (TFEC, DCVC and TFEC; approx. 2700:1) than in the presence of β-chloro-L-alanine (approx. 3850:1). Taken together, the results suggest that rat liver mitAspAT is subjected to a 'two-pronged' attack in the presence of the cysteine S-conjugates, i.e. (a) aminoacrylate attack on an active-site residue (including, but not necessarily limited to, PLP); and (b) thioacylation of a susceptible residue.

The biphasic nature of the progress curve for inactivation of mitAspAT by TFEC in the absence of added 2-oxoglutarate (Figure 1) is explained by transamination competing with the β-lyase reaction at a low ratio (< 1:100). Transamination of the cysteine S-conjugate with the cofactor will eventually convert the PLP form of the enzyme (active in transamination reactions and β-lyase reactions) into the PMP form (active in transamination reactions only). Because the PMP form cannot catalyse a β-lyase reaction, the enzyme is spared further inactivation. Under the conditions shown in Figure 1 (5.4 μg/ml of mitAspAT in the assay mixture), approx. 25% of the enzyme is in the PMP form at 1 h, and is resistant to further inactivation. Pyruvate formed in the β-lyase reaction (or 'oxo' TFEC formed by transamination) is theoretically capable of converting the PMP form of the enzyme back into the PLP form, restoring the β-lyase activity and concomitant syncatalytic inactivation. However, pyruvate is such a poor substrate that the transamination between this 2-oxo acid and the PMP form of the enzyme is slow. It was noted, however, that, at much higher enzyme concentrations (100 μg/ml), the relative rate of syncatalytic inactivation in the presence of TFEC was increased and residual enzyme activity was less (approx. 5%; results not shown). When more enzyme is present, proportionately more pyruvate and 'oxo' TFEC are produced, which will result in more favourable transamination of the PMP form of the enzyme back to the PLP form, and more extensive inactivation (cf. [26]). If this hypothesis is correct, then addition of 2-oxoglutarate should prevent the build up of the PMP form of mitAspAT in the presence of TFEC, and the enzyme should be inactivated in a monophasic manner. This was found to be the case (Figure 1).

As discussed in the Introduction, the kidney is especially sensitive to toxic cysteine S-conjugates. Within the kidney, the S_3 segment of the proximal tubule is the most sensitive. In rats, the kidney and brain have high levels of mitAspAT, although somewhat less than in the liver and heart [43]. Total AspAT, but not mitAspAT or cytAspAT, has been measured in microdissected regions of the rat nephron [44]. The S_3 region of the proximal tubules had moderate activity of 'total' AspAT [44]. Several authors have commented that the toxicity of cysteine S-conjugates to a given organ will depend not only on the presence of a cysteine S-conjugate β-lyase in the mitochondria, but also on other factors, such as cellular uptake, mitochondrial uptake, acylation and deacylation (e.g. [4,5]). Evidently, the susceptibility of various organs to toxic cysteine S-conjugates cannot be explained solely by the relative distribution of mitAspAT. Factors that lead to the uptake of the toxic cysteine S-conjugate (or the corresponding glutathione- or *N*-acetyl-cysteine S-conjugates; see the Introduction), accumulation of toxic cysteine S-conjugates in the mitochondrial matrix, proximity of the toxicants to target proteins, protection from natural substrates and the presence of other mitochondrial cysteine S-conjugate β-lyases may also have a role.

An interesting, but presently unresolved, question is why mitochondrial, but not cytosolic, proteins in kidney become labelled by a fragment derived from TFEC *in vivo* when PLP-dependent enzymes capable of catalysing a cysteine S-conjugate β-lyase reaction *in vitro* (e.g. cytosolic glutamine transaminase K) are well represented in the kidney cytosol [6]. Possibly, the mitochondrial uptake system for TFEC has greater affinity and/or capacity than that for uptake across the cell membrane, so that the concentration of TFEC reaches a greater level in mitochondria than in cytosol. Alternatively: (a) TFEC may be carried through the cytosol in such a manner that it does not come in contact with the cytosolic cysteine S-conjugate β-lyases; (b) the natural substrates of the cytosolic cysteine S-conjugate β-lyases prevent productive binding of TFEC as a lyase substrate; or (c) TFEC converts the cytosolic glutamine transaminase K into an unproductive PMP form, which can only be slowly converted back into the productive PLP form because of the low level of the natural 2-oxo acid substrates (2-oxo-4-methiolbutyrate and phenylpyruvate). (Because transamination competes effectively with β elimination, glutamine transaminase K has an absolute requirement for addition of 2-oxo acids in order to promote cysteine S-conjugate β-lyase reactions [45].) The exact mechanism for the selective thioacylation of kidney

mitochondrial proteins by a fragment derived from TFEC must await further studies. Whatever the mechanism, the passage of TFEC into the mitochondria ensures that it will be available as a β-lyase substrate for several PLP enzymes, including mitAspAT.

While there is much evidence that mitAspAT is bound to the inner mitochondria membrane, where it forms a supramolecular complex with OGDHC [35,46], there is also evidence that mitAspAT is present in plasma membranes of certain cell types, where it has been identified as a fatty acid-binding protein ('FABPpm') [47]. Notably, the enzyme is prominent in kidney membranes [47]. Whether the extramitochondrial location of mitAspAT contributes to the selective sensitivity of kidney (in general) and of the renal cytosol (more specifically) to toxic halogenated cysteine S-conjugates remains to be determined.

Conclusions

The present study shows that mitAspAT can catalyse a cysteine S-conjugate β-lyase reaction with toxic halogenated cysteine S-conjugates, but, at the same time, it is slowly inactivated. Although the rate of the cysteine S-conjugate β-lyase reaction is relatively low compared with the optimal rate of transamination with natural substrates, the very high endogenous levels of mitAspAT in mitochondria suggest that this enzyme may be a significant (but not the only) factor in the mitochondrial toxicity associated with certain halogenated cysteine S-conjugates. The very high concentration of mitAspAT in the inner mitochondrial membrane and its close association with OGDHC provides an explanation for previous findings that kidney OGDHC is especially vulnerable to inactivation when rats are challenged with the nephrotoxic halogenated cysteine S-conjugate TFEC, and that mitAspAT is itself a target. Although mitAspAT is widely distributed in tissues, the selective vulnerability of the kidney to toxic halogenated cysteine S-conjugates is explained in part by an enterohepatic circulation that ensures much greater exposure of the proximal tubules to the toxicant than other cell types.

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