L-Alanine–glyoxylate aminotransferase II of rat kidney and liver mitochondria possesses cysteine S-conjugate β-lyase activity: a contributing factor to the nephrotoxicity/hepatotoxicity of halogenated alkenes?

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Several halogenated alkenes are metabolized in part to cysteine S-conjugates, which are mitochondrial toxicants of kidney and, to a lesser extent, other organs. Toxicity is due to cysteine S-conjugate β-lyases, which convert the cysteine S-conjugate into pyruvate, ammonia and a reactive sulphur-containing fragment. A section of the human population is exposed to halogenated alkenes. To understand the health effects of such exposure, it is important to identify cysteine S-conjugate β-lyases that contribute to mitochondrial damage. Mitochondrial aspartate aminotransferase [Cooper, Bruschi, Iriarte and Martinez-Carrion (2002) Biochem. J. **368**, 253–261] and mitochondrial branched-chain aminotransferase [Cooper, Bruschi, Conway and Hutson (2003) Biochem. Pharmacol. **65**, 181–192] exhibit β-lyase activity toward *S*-(1,2-dichlorovinyl)-L-cysteine (the cysteine S-conjugate of trichloroethylene) and *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (the cysteine S-conjugate of tetrafluoroethylene). Turnover leads to eventual inactivation of these enzymes. Here we report that mitochondrial L-alanine–glyoxylate aminotransferase II, which, in the rat, is most active in kidney, catalyses cysteine S-conjugate β-lyase reactions with *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(benzothiazolyl-L-cysteine); turnover leads to inactivation. Previous workers showed that the reactive-sulphur-containing fragment released from *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-L-cysteine is toxic by acting as a thioacylating agent – particularly of lysine residues in nearby proteins. Toxicity, however, may also involve 'self-inactivation' of key enzymes. The present findings suggest that alanine–glyoxylate aminotransferase II may be an important factor in the well-established targeting of rat kidney mitochondria by toxic halogenated cysteine S-conjugates. Previous reports suggest that alanine–glyoxylate aminotransferase II is absent in some humans, but present in others. Alanine–glyoxylate aminotransferase II may contribute to the bioactivation (toxification) of halogenated cysteine S-conjugates in a subset of individuals exposed to halogenated alkenes.

Key words: L-alanine–glyoxylate aminotransferase II, β-chloro-L-alanine, cysteine S-conjugate β-lyase activity, *S*-(1,2-dichlorovinyl)-L-cysteine, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, toxicity of halogenated alkenes.

INTRODUCTION

Several halogenated alkenes (e.g. trichloroethylene, tetrachloroethylene and tetrafluoroethylene) are in widespread industrial and commercial use. Trichloroethylene causes renal and liver tumours in experimental animals, including rats (e.g. [1,2]). Prolonged exposure to high levels of trichloroethylene results in an elevated risk of kidney cancer in human beings [3–5]. Tetrafluoroethylene, the precursor of Teflon™, is a nephrotoxicant, nephrocarcinogen and hepatocarcinogen in rodents [6,7]. Tetrachloroethylene (perchloroethylene, perc) is a weak nephrocarcinogen in rats [8]. Trichloroethylene is a major environmental contaminant in some parts of the U.S.A. [9]. Because of the potential for human exposure to halogenated alkenes in the workplace and general environment, it is important to characterize their bioactivation mechanisms.

The toxicity of halogenated alkenes is due in part to the formation of the corresponding cysteine S-conjugates (e.g. [10]). *S*-(1,2-Dichlorovinyl)-L-cysteine (DCVC) and *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC) are the cysteine S-conjugates derived from trichloroethylene and tetrafluoroethylene respectively. These halogenated cysteine S-conjugates are converted into pyruvate (via aminoacrylate), ammonium and a reactive-sulphur-containing fragment by the action of cysteine S-conjugate β-lyases. The net reaction is shown in eqn (1), where R=F_2CHCF_2- and ClHC=Cl- for TFEC and DCVC respectively:

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

The toxicity of DCVC has been demonstrated in isolated rat kidney cells (e.g. [11]), isolated human kidney proximal tubules [12]

Abbreviations used: AlaAT, alanine aminotransferase; AGAT, L-alanine–glyoxylate aminotransferase (unspecified isoenzyme); AGAT I, L-alanine–glyoxylate aminotransferase I; AGAT II, L-alanine–glyoxylate aminotransferase II; AspAT, aspartate aminotransferase; $BCAT_c$, cytosolic branched-chain aminotransferase; $BCAT_m$, mitochondrial branched-chain aminotransferase; BTC, benzothiazolyl-L-cysteine; cyt, cytosolic; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DTT, dithiothreitol; ipptn., immunoprecipitation; GTK, glutamine transaminase K; LDH, lactate dehydrogenase; mit, mitochondrial; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

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and in primary cultures of human proximal tubules [13]. TFEC is nephrotoxic and weakly hepatotoxic in rats [14]. The toxicity of halogenated cysteine S-conjugates is due in part to the high reactivity of the eliminated sulphur-containing fragment. The sulphur-containing fragments derived from DCVC and TFEC apparently break down to a thioketene [15] and dihalothionoacetyl fluoride [10,16] respectively, both of which are thioacylating agents of macromolecules – especially of lysine residues in proteins (e.g. [17]; for reviews, see, e.g., [18–20]).

Mitochondria are targeted by toxic halogenated cysteine S-conjugates (reviewed in [20]), and at least six mitochondrial proteins are thioacylated in kidney mitochondria after rats are administered TFEC [21–23]. Several mitochondrial enzymes of energy metabolism are inactivated in intact kidney [21–23] and PC12 [24] cells exposed to TFEC. In order to understand the mechanisms underlying the vulnerability of mitochondria to halogenated cysteine S-conjugates, it is important to characterize mitochondrial cysteine S-conjugate β-lyases. Mitochondrial cysteine S-conjugate β-lyases identified thus far include a rat kidney/liver high-M_r lyase [25], rat mitochondrial aspartate aminotransferase (mitAspAT) [26] and human mitochondrial branched-chain aminotransferase ($BCAT_m$) [27].

Rat kidney and liver contain a mitochondrial aminotransferase designated alanine–glyoxylate aminotransferase II [28,29] (AGAT II; eqn 2):

$$\text{L-Alanine} + \text{glyoxylate} \rightarrow \text{pyruvate} + \text{glycine} \quad (2)$$

Rat kidney AGAT II is a homotetramer (subunit M_r of the precursor protein $\approx 57\,000$; subunit M_r of the mature protein $\approx 53\,000$] that contains pyridoxal 5′-phosphate (PLP) as a cofactor [30]. AGAT II catalyses transamination of the relatively elongated amino acids N^G,N^G-dimethyl-L-arginine, N^G,N'^G-dimethyl-L-arginine, N^G-monomethyl-L-arginine, N^ω-nitroso-L-arginine and L-α-aminobutyrate with pyruvate or glyoxylate [29,31] and transamination of the elongated aldehyde γ,δ-dioxovalerate with L-alanine [32]. The enzyme also catalyses an α-elimination reaction with (*R*,*S*)-α-fluoro-β-alanine and a β-elimination reaction with β-chloro-L-alanine [33]. The enzyme is syncatalytically inactivated by β-chloro-L-alanine, but not by (*R*,*S*)-α-fluoro-β-alanine [33]. Given the ability of AGAT II to (a) productively accommodate relatively elongated substrates at its active site and (b) catalyse a β-elimination reaction with an amino acid with a good leaving group at the β-position (i.e. β-chloro-L-alanine), the possibility existed that AGAT II might also be a mitochondrial cysteine S-conjugate β-lyase. The present work confirms this notion. Moreover, as previously noted with β-chloro-L-alanine [33], turnover of the cysteine S-conjugates was shown to lead to inactivation of AGAT II. The toxicological implications of the findings are discussed.

EXPERIMENTAL

Reagents and enzymes

EGTA, Hepes, dithiothreitol (DTT), BSA (fatty-acid-free), 2,4-dinitrophenylhydrazine, ammediol (2-amino-2-methylpropane-1,3-diol), Tris, β-chloro-L-alanine hydrochloride, DEAE-cellulose, NAD^+, NADH, pig heart malate dehydrogenase [910 units/mg in 50 % (v/v) glycerol/50 mM potassium phosphate buffer, pH 7.5; 5.6 mg/ml], rabbit muscle lactate dehydrogenase [LDH, type XXXIX; 720 units/mg of protein in 50 % (v/v) glycerol; 2.9 mg/ml], *Bacillus subtilis* alanine dehydrogenase (40 units/mg as a suspension in 2.4 M $(NH_4)_2SO_4$, PLP, lyophilized Protein A–agarose, Tricine, Coomassie Blue, sodium pyruvate, sodium glyoxylate, L-alanine, L-phenylalanine, and sodium 2-oxo-4-methiolbutyrate were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.). Pre-cast 10–20 % (w/v) polyacrylamide gels and SeeBlue® molecular-mass markers were from Invitrogen (Carlsbad, CA, U.S.A.). TFEC [17] and the acetate salt of BTC (benzothiazolyl-L-cysteine) [25] were generously provided by Dr Sam Bruschi (Department of Medicinal Chemistry, University of Washington, Seattle, WA, U.S.A.). DCVC was kindly given by Dr James L. Stevens (Lilly Research Laboratories, Greenfield, IL, U.S.A.). Stock solutions of β-lyase substrates, namely 20 mM BTC in 100 mM ammediol/HCl buffer, pH 9.0, 20 mM DCVC in 100 mM Tris/HCl, pH 8.0, and 100 mM TFEC in 100 mM Tris/HCl, pH 8.0, and 100 mM β-chloro-L-alanine hydrochloride (neutralized with NaOH) in distilled water were stored at -20 °C. In some cases, TFEC (100 mM) was dissolved in 100 mM potassium phosphate buffer, pH 8.0. The pH of the BTC solutions were adjusted to 7.8 with 1 M HCl before use. Hydroxyapatite was from Bio-Rad (Hercules, CA, U.S.A.). Sephacryl S-200 was from Pharmacia (Piscataway, NJ, U.S.A.).

Enzyme measurements

The standard AGAT II assay was similar to that described by Okuno et al. [29], except that the procedure was modified for 96-well-plate analyses. The reaction mixture (50 μl) contained 40 mM L-alanine, 10 mM sodium glyoxylate, 10 μM PLP, 5 mM DTT, 100 mM potassium phosphate buffer, pH 8.0, and enzyme. After incubation at 37 °C, the reaction was stopped by the addition of 150 μl of 0.75 M Tris/HCl, pH 8.0, containing 0.1 mM NADH and 5 μg/ml LDH. The disappearance of NADH was monitored at 340 nm (ε 6230 $M^{-1} \cdot cm^{-1}$) after addition of the 'stop' reagent. Under these conditions, the reduction of pyruvate is complete in 5 min at 37 °C. (Both glyoxylate and pyruvate are excellent substrates of LDH, but discrimination between the two is afforded by addition of Tris, which forms an adduct with glyoxylate, but not with pyruvate [34].) The blank consisted of complete reaction mixture plus enzyme, except that glyoxylate was omitted. Pilot experiments showed that the AGAT II reaction catalysed by small aliquots (2–5 μl) of crude rat kidney homogenates is linear for at least 20 min under the conditions of the assay, but because of high activity, incubations of 5 min were typically used. For purified and semi-purified preparations of AGAT II, a modification of the more convenient, but less sensitive assay described by Porter et al. [33] was used. The reaction mixture (200 μl) contained 20 mM L-alanine, 10 mM sodium glyoxylate, 10 μM PLP, 100 mM potassium phosphate buffer, pH 8.0, and enzyme. The increase in absorbance at 220 nm was continuously recorded at 37 °C. At this wavelength, the absorbance of glyoxylate is small (ε 80 $M^{-1} \cdot cm^{-1}$), whereas that of pyruvate is considerably greater (ε 1050 $M^{-1} \cdot cm^{-1}$) [33]. The increase in absorbance is linear for at least 1 h in the presence of 5 μl of highly purified enzyme.

Glutamine transaminase K (GTK) was assayed as described [24]. GTK (identical with kynurenine aminotransferase I) is a freely reversible glutamine/methionine/aromatic amino acid aminotransferase [35]. The standard assay measures transamination between L-phenylalanine and 2-oxo-4-methiolbutyrate. mitAspAT activity was measured in a reaction mixture (0.2 ml) containing 10 mM L-aspartate, 6 mM 2-oxoglutarate, 100 mM Hepes, pH 7. 5, 0.1 mM NADH and 0. 6 μg of malate dehydrogenase, as described previously [26]. Cysteine S-conjugate β-lyase activity was measured by a slight modification of the method described previously [25]. The standard cysteine S-conjugate β-lyase reaction mixture contained 20 mM TFEC,

10 μM PLP, 100 mM potassium phosphate buffer, pH 8.0, and enzyme in a volume of 20 μl. After incubation at 37 °C in a small snap-top tube, the reaction was terminated by the addition of 20 μl of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After 10 min incubation at room temperature, 160 μl of 1 M NaOH was added and the absorbance at 430 nm was read within the next 2 min. The molar absorption coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions is 15 000. In other cases, TFEC in the β-lyase reaction mixture was replaced by DCVC, BTC or β-chloro-L-alanine. Some non-enzymic formation of pyruvate occurs with TFEC (and β-chloro-L-alanine) in the presence of PLP (typically 1–5 nmol/h per 20 μl reaction mixture). The blank for the β-lyase reactions was the complete reaction mixture plus boiled enzyme. Because most cysteine S-conjugate β-lyases (including AGAT II) are eventually inactivated by β-lyase substrates, incubation times were generally short (0.5–3 min) to ensure that initial reaction rates were linear.

All spectrophotometric measurements were carried out with a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). A unit of enzyme activity for all enzymes investigated here is that which catalyses the formation of 1 μmol of product/min at 37 °C. For enzyme-activity measurements, the period of incubation is given in the Results section.

Rats

Six-month-old male Fisher 344 × Brown Norway F_1 rats were used. The animals were fed *ad libitum* and had full access to water. The rats were killed by decapitation. The experiments reported here were approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee (Protocol no. 0005-713A).

Purification of AGAT II from rat kidney

The procedure was a modification of that used by Okuno et al. [29]. All procedures were carried out at 0–4 °C, unless otherwise stated, and all centrifugations were at 10 000 ***g*** for 30 min. The homogenization buffer (HB) consisted of 5 mM potassium phosphate buffer, pH 7.5, 1 mM sodium pyruvate, 0.1 mM PLP, 5 mM DTT and 10 % (v/v) glycerol. Elution buffer 1 (EB1) was 5 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT. Elution buffer 2 (EB2) was 5 mM potassium phosphate buffer, pH 6.5, containing 2 mM DTT. Rat kidneys (24.2 g) were homogenized in 100 ml of 0.25 M sucrose containing 1 ml of protease-inhibitor cocktail (Sigma) by means of a hand-held glass-to-glass homogenizer. The crude mitochondrial pellet, obtained by centrifugation of the homogenate at 1000 ***g*** for 10 min, was used as the source of AGAT II. The mitochondrial pellet was frozen at − 80 °C, thawed, suspended in 100 ml of HB, and sonicated for 2 min at 70 % maximal power using a Vibra-Cell (Sonics & Materials, Danbury, CT, U.S.A.). Insoluble material was removed by centrifugation. The supernatant was rapidly heated in a 95 °C water bath to 60 °C and held at this temperature for 3 min, followed by rapid cooling in an ice bath. After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant to 30 % saturation. After gentle stirring for 60 min, the precipitate was removed by centrifugation and discarded. The supernatant was then adjusted to 80 % saturation with $(NH_4)_2SO_4$. After gentle stirring for 60 min, the precipitate was removed by centrifugation. The pellet was dissolved in a minimal volume of HB and dialysed against 2 × 5 litres of HB. The inactive precipitate was removed by centrifugation. The supernatant was added to the top of a hydroxyapatite column (10 cm × 1.5 cm) equilibrated with EB1. The column was eluted with 500 ml of EB, followed by a 1 litre linear gradient of 5–200 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT. Active fractions were combined, dialysed against 5 litres of HB, and then added to the top of a DEAE-cellulose column equilibrated with EB1. The column was eluted with 500 ml of EB, followed by a 1 litre linear gradient of 5–200 mM potassium phosphate buffer containing 2 mM DTT, pH 7.5. $(NH_4)_2SO_4$ was added to the combined active fractions to 80 % saturation. The precipitate was taken up in minimal volume of EB1 and added to the top of a Sephacryl S-200 (60 cm × 2.5 cm) column. The column was eluted with EB1. The active fractions were combined and added to a second hydroxyapatite column (10 cm × 1.5 cm) equilibrated with EB2. The column was eluted with 500 ml of EB2, followed by a 440 ml linear gradient of 5–200 mM potassium phosphate buffer, pH 6.5, containing 2 mM DTT. The active fractions were combined and concentrated by ultrafiltration. After the protein content was determined, glycerol and PLP were added to final concentrations of 30 % (v/v) and 50 μM respectively. The purified enzyme (1.5 ml) was stored at − 20 °C. The preparation had no detectable GTK, alanine aminotransferase (AlaAT) or AspAT activities. The final preparation was estimated to be about 80 % pure as judged by SDS/PAGE (Figure 1). A major band with $M_r \approx 53\,000$ (AGAT II) and a contaminating band with an M_r of $\approx 70\,000$ were observed. The yield (0.4 mg) and specific activity (40 units/mg) of purified enzyme are comparable with literature values of ≈ 1–2 mg/100 g wet weight of rat kidney and ≈ 30–160 units/mg respectively [29,31,33]. The procedure was repeated twice with comparable results. It should be noted that semi-purified preparations of AGAT II are relatively unstable in the absence of glycerol and PLP, losing all activity in 1 week at 4 °C. The purification must therefore be carried out as quickly as possible.

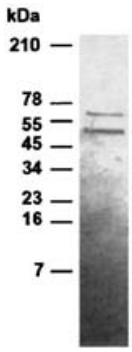

Figure 1 Tricine/SDS/PAGE of purified AGAT II

The values refer to molecular-mass markers run in an adjacent lane. The amount of enzyme applied to the gel was 20 μg.

Preparation of highly purified rat liver mitochondria

A standard procedure [36] for the isolation of mitochondria from large quantities of liver was adapted for the isolation of mitochondria from a single rat liver (about 8–10 g wet weight). All procedures were carried out at 0–4 °C in a coldroom. The liver was rapidly removed and placed in a small beaker containing 40 ml of ice-cold isolation buffer [300 mM sucrose/0.5 mM EGTA/10 mM Hepes/0.5 % (w/v) BSA, pH 7.4 (adjusted with KOH)]. The liver was minced with scissors, washed with isolation buffer and homogenized in a loose-fitting Dounce homogenizer (100 ml volume) at a tissue/buffer ratio of 1 g wet weight/8–10 ml. Isolation of the mitochondria was carried out by a differential centrifugation

procedure using a Beckman centrifuge and a JA-20 rotor. The homogenate (H) was centrifuged for 10 min at 1000 ***g***. The pellet containing blood and cell fragments was discarded. The supernatant fraction (S) containing the mitochondria was carefully removed and centrifuged for 10 min at 10000 ***g***. Two aliquots (about 1 ml of each) from the top and middle supernatant (cytosolic) fractions were removed (C1 and C2 respectively). The remainder of the supernatant was discarded. Lipid lining the interior of the centrifuge tube was carefully wiped away with a tissue. The pellet, which contained crude mitochondria, was resuspended in 5 ml of isolation buffer using a loose-fitting 10 ml Dounce homogenizer. The crude mitochondrial suspension was placed in a centrifuge tube and the total volume was adjusted to 40 ml with isolation buffer. The suspension was centrifuged for 10 min at 10000 ***g***. The supernatant was discarded and the interior of the centrifuge tube was wiped clean with tissue paper. The mitochondrial pellet contained two layers – one light-brown in colour (top) and the other dense brown (bottom). The light-brown layer was suspended by addition of 5 ml of washing buffer (isolation buffer minus EGTA and BSA), followed by gentle shaking. The suspension was discarded. The dense-brown pellet was resuspended in 5 ml of washing buffer in a 10 ml-volume Dounce homogenizer. The suspension was placed in a centrifuge tube and the total volume was adjusted with washing buffer to 40 ml. The final centrifugation step was carried out for 10 min at 10000 ***g***. The top surface of the dense-brown pellet was washed twice with washing buffer (to eliminate possible contamination with the light-brown mitochondrial fraction) and the interior of the tube was wiped with tissue paper. The final pellet was weighed and re-suspended in washing buffer in a 5 ml-volume Dounce homogenizer at a pellet/buffer ratio of 400 mg:1 ml.

Mitochondria (M) prepared using this procedure and kept on ice are stable (i.e. maintained respiration in the presence of suitable substrates) for over 24 h after isolation.

Immunoprecipitation of rat kidney AGAT II

Antiserum to rat AGAT II was prepared in rabbits as described in [29]. Titration of crude kidney mitochondrial homogenates with antiserum showed that 25 μl of antiserum was sufficient to bind 100 munits of AGAT II (results not shown). To precipitate AGAT II, antigen and antiserum (ratio 100 munits of AGAT II to 25 μl of antiserum) were mixed in 1 ml of 10 mM potassium phosphate buffer, pH 7.4. After leaving at room temperature for 1 h, 10 mg of Protein A–agarose (previously rinsed with 10 mM potassium phosphate buffer, pH 7.4) was added per 50 μl of antiserum. After a further 30 min incubation at room temperature, the insoluble material was removed by centrifugation at 10000 ***g*** for 2 min. The insoluble material was resuspended and centrifuged (10000 ***g***, 2 min) four times with 10 mM potassium phosphate buffer, pH 7.4). The insoluble material was finally suspended in 10 mM potassium phosphate buffer, pH 7.4, at a ratio of 840 munits of immunoprecipitated enzyme/ml. The ipptd. enzyme retains activity (see below). Controls contained Protein A–agarose exposed to immune serum/boiled kidney mitochondrial homogenate ('Control Protein A'). For measurement of AGAT activity in the immunoprecipitate, the standard AGAT reaction mixture (50 μl) contained 10 μl of the suspension in a small snap-top tube. After incubation at 37 °C, 0.18 ml of water was added, the mixture was cooled to 2 °C, and the suspension was centrifuged at 10000 ***g*** for 2 min. An aliquot (0.19 ml) of the clear supernatant was removed and the absorbance due to pyruvate formation was measured at 220 nm in a 96-well plate analyser. For measurement of β-lyase activities, the reaction mixture (20 μl) contained 100 mM potassium phosphate buffer, pH 8.0, 10 μM PLP, β-lyase substrate and 10 μl of the suspension. After incubation at 37 °C in a small snap-top tube, the reaction was terminated by the addition of 20 μl of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After a further 5 min incubation, 0.16 ml of 1 M NaOH was added. The suspension was centrifuged at 10000 ***g*** for 2 min and the absorbance of 0.18 ml of the clear supernatant solution was measured at 430 nm in a 96-well plate analyser.

Determination of transamination versus β-elimination in the presence of purified AGAT II and TFEC

Purified enzyme (20 μl; 210 m-units) was incubated with 20 mM TFEC, 10 μM PLP and 100 mM Tris/HCl buffer, pH 8.0, in a final volume of 0.1 ml. After incubation for 6 h at 37 °C in a small snap-top tube, a 10 μl aliquot was withdrawn and analysed for pyruvate formation by the 2,4-dinitrophenylhydrazine method. An aliquot (70 μl) of the remaining fraction was transferred to a 96-well plate and analysed for L-alanine formation. The volume of liquid in the well was adjusted to 0.2 ml with 40 μl of 1 M carbonate/bicarbonate buffer, pH 10.4, 20 μl of 20 mM NAD^+ and 70 μl of water. The absorbance at 340 nm was determined. Thereafter alanine dehydrogenase (2 μl; 200 m-units) was added and the absorbance was read 5 min later. Only a small change in absorbance ($\approx$0.02) was noted, which was no different from a blank reaction mixture containing 20 mM TFEC and 10 μM PLP that had previously been incubated for 6 h. As a check that the alanine dehydrogenase was active, addition of 2 μl of 1 mM L-alanine to the 6 h TFEC/AGAT II plus alanine dehydrogenase reaction mixture resulted in a rapid increase in absorbance at 340 nm (results not shown). An absorbance change of 0.02 (equivalent to 1.25 nmol of L-alanine oxidized to pyruvate) relative to the blank would have been easily detectable in the assay mixture containing TFEC and AGAT II incubated for 6 h.

Determination of kinetic constants of purified AGAT II

The K_m and V_{max} values for L-alanine were determined in 0.2 ml reaction mixtures containing 10 mM glyoxylate, 10 μM PLP, 100 mM potassium phosphate buffer, pH 8.0, various L-alanine concentrations and enzyme (2.5 μl; 66.7 munits), at 37 °C. The absorbance due to pyruvate was continuously measured at 220 nm. The K_m and V_{max} values for the β-lyase substrates were determined in 0.2 ml reaction mixtures containing 10 μM PLP, 100 mM potassium phosphate buffer, pH 8.0, and enzyme (5–10 μl; 133–267 munits), at 37 °C. The reaction mixture was warmed to 37 °C, enzyme was added and the rate of increase of absorbance at 220 nm was recorded for 30 s when β-chloro-L-alanine was the substrate and for 2 min when TFEC or BTC were the substrates. K_m and V_{max} values were determined from Lineweaver–Burk plots.

Protein measurements

Protein concentrations were determined by the micro-Biuret method using the Sigma Protein Assay Kit and BSA as a standard.

Statistical analysis

For determinations where n is $\geqslant 3$, means $\pm$ S.E.M. are reported. Statistical comparisons were carried out using the Mann–Whitney U-test; a P value of $\leqslant 0.05$ was considered significant.

Table 1 Comparison of TFEC β-lyase, β-chloro-L-alanine β-lyase and AGAT activities of crude rat kidney mitochondria, crude rat kidney cytosol and purified rat kidney AGAT II

The volumes of the crude mitochondrial and cytosolic fractions (prepared from 24.2 g of kidney) were 110 and 120 ml respectively. Total protein was 1770 and 1800 mg respectively. The AGAT activities in the mitochondrial and cytosolic fractions were 334 and 25.2 units respectively. Results are the average of three determinations. The concentration of TFEC and β-chloro-L-alanine was 20 mM.

	Specific activity (units/mg of protein)				
	TFEC β-lyase (A)	β-Chloro-L-alanine β-lyase (B)	AGAT (C)	A/C	B/C
Mitochondrial fraction	0.025 ± 0.003	0.094 ± 0.01	0.189 ± 0.005	0.132	0.497
Cytosolic fraction	0.039 ± 0.002	0.177 ± 0.012	0.014 ± 0.004	2.79	12.6
Purified AGAT II	1.20 ± 0.15	8.1 ± 0.13	40.1 ± 0.3	0.030	0.201

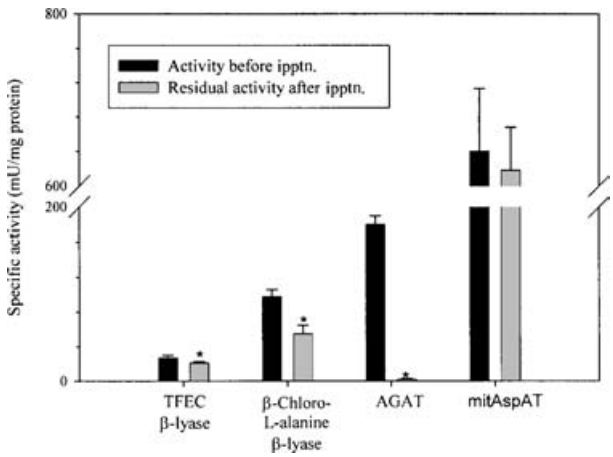

Figure 2 Immunoprecipitation (ipptn.) of TFEC β-lyase, β-chloro-L-alanine β-lyase and AGAT activities from rat kidney mitochondria

Crude kidney mitochondria were obtained as described in the Experimental section from four separate kidneys. The mitochondrial preparations were freeze–thawed and sonicated. AGAT II in the disrupted mitochondria was precipitated with 25 μl of immune serum per 100 munits (mU) of enzyme activity and 10 mg of Protein A–agarose per 50 μl of antiserum. Enzyme activities in the disrupted mitochondria before and after addition of immune serum were determined as described in the Experimental section. mitAspAT activity was determined as a control. The assays were determined in triplicate for each of the four kidney preparations. The calculation of specific activities after the addition of immune serum is based on the assumption that < 1 % of the total protein in the mitochondrial fraction is immunoprecipitated (from a comparison of the specific activities of AGAT II in the mitochondrial fraction versus that in the purified enzyme preparation; Table 1). The concentration of TFEC and β-chloro-L-alanine in these experiments was 20 mM. Statistical significance: *different from before ipptn. with $P = 0.05$.

RESULTS

Contribution of AGAT II to β-lyase activity in crude rat kidney mitochondria when TFEC and β-chloro-L-alanine are used as substrates

Table 1 shows that purified AGAT II exhibits β-lyase activity toward TFEC and β-chloro-L-alanine. The ratios of TFEC β-lyase to AGAT specific activities for the mitochondrial fraction and purified enzyme are 0.132 and 0.030 respectively (Table 1). Therefore, under the conditions used for assay, ≈23 % [i.e. (0.030/0.132) × 100] of the TFEC β-lyase activity in the crude rat kidney mitochondrial preparation is due to AGAT II. A similar calculation shows that ≈40 % of the β-chloro-L-alanine β-lyase activity of the rat kidney mitochondria is due to AGAT II (Table 1). The specific activities of TFEC β-lyase and β-chloro-L-alanine β-lyase are greater in the crude rat kidney cytosol than in the crude mitochondrial fraction (Table 1). Thus contamination of the mitochondrial fraction with cytosolic proteins could lead to an overestimation of β-lyase activities in the mitochondrial fraction. However, only about 5 % of the total LDH activity (cytosolic marker) in the kidney homogenates was in the mitochondrial fraction, suggesting that the contribution of cytosolic enzymes to the TFEC β-lyases in the mitochondrial fraction is low (results not shown). Only about 7 % of AGAT activity (an exclusively mitochondrial activity in rat kidney *in vivo*; see the Discussion) was in the crude cytosolic fraction (Table 1). The results suggest that, although the rat kidney mitochondrial preparation obtained here was relatively crude, it contained most of the AGAT II activity.

A significant (≈22 %) amount of the total TFEC β-lyase activity of the rat kidney mitochondria was precipitated by the AGAT II immune serum under conditions that removed almost all the AGAT activity (Figure 2). Under these conditions, about 38 % of the β-chloro-L-alanine β-lyase activity of the rat kidney mitochondria was precipitated by the immune serum (Figure 2). As a control, the activity of mitAspAT was measured in the rat kidney mitochondrial fraction before and after treatment with immune serum. No significant difference in the specific activity of mitAspAT in the mitochondrial fraction was noted after treatment with the AGAT II immune serum (Figure 1). Taken together, the results suggest that, under the conditions of the assay, ≈20 % of the TFEC β-lyase activity and ≈40 % of the β-chloro-L-alanine β-lyase activity in the crude rat kidney mitochondria is due to AGAT II.

Aminotransferase and β-lyase activities of AGAT II immunoprecipitated from a crude rat kidney mitochondrial homogenate

The experiment described in Figure 2 shows that enzyme (840 munits) immunoprecipitated directly from a crude rat kidney mitochondrial homogenate retains its ability to catalyse transamination between L-alanine and glyoxylate. The AGAT activity (367 munits) in the 1 ml suspension calculated from the data in Figure 2 (solid bar) shows that the immunoprecipitated enzyme is at least 44 % as active as free enzyme. Moreover, the immunoprecipitated enzyme also retains β-lyase activity with TFEC and β-chloro-L-alanine (Figure 2). Immunoprecipitated AGAT II was also noted to catalyse β-lyase reactions with DCVC and BTC (Figure 3) (in a recent review [20], AGAT-II-catalysed β-lyase activity with BTC was incorrectly listed as − instead of +).

Inactivation of AGAT II by β-lyase substrates: calculation of ratio of pyruvate formed to enzyme monomer inactivated

Table 2 shows that highly purified AGAT II is inactivated in the presence of β-lyase substrates. Inactivation is more rapid with β-chloro-L-alanine than with TFEC or BTC. The amount (nmol) of pyruvate formed from β-chloro-L-alanine, TFEC and BTC per nmol of enzyme subunit inactivated was calculated to be 1690 ± 110, 1450 ± 120 and 1800 ± 190 respectively. The amount of pyruvate formed from TFEC was significantly less than that formed from β-chloro-L-alanine.

Figure 3 shows that immunoprecipitated AGAT II is also inactivated by β-lyase substrates and that inactivation with β-chloro-L-alanine is more rapid than with the cysteine S-conjugates. As noted in the Experimental section, our data suggest that

Table 2 Inactivation of purified rat kidney AGAT II by β-lyase substrates

The reaction mixture (0.2 ml) contained β-lyase substrate, 100 mM potassium phosphate buffer, pH 8.0, 10 μM PLP and 134 munits of enzyme (50.2 pmol of enzyme subunit); incubation was at 37 °C. In each case, the rate of pyruvate formation (measured by the increase in absorbance at 220 nm) decreased in a first-order manner, from which the $t_{1/2}$ for inactivation was calculated. In a separate experiment in which the reaction mixtures were incubated in a small close-capped tube for 6 h (at which time AGAT II is completely inactivated), the amount of pyruvate generated from β-chloro-L-alanine, TFEC and BTC averaged 85, 72.8, 90.1 nmol respectively. The average change in absorbance at 220 nm was 0.202, 0.173 and 0.214 respectively. In a control containing 20 mM L-alanine in place of β-lyase substrate, no loss of activity was noted after the 6 h incubation.

Substrate	Concn. (mM)	$t_{1/2}$ (min)	Pyruvate formed/ monomer inactivated
β-Chloro-L-alanine	20	4.0 ± 0.3	1690 ± 110
TFEC	20	18 ± 2	1450 ± 120*
BTC	5	30 ± 3	1800 ± 190

* Significantly different from the value obtained with β-chloro-L-alanine with $P = 0.05$.

Table 3 Relative distribution of TFEC β-lyase and AGAT activities in rat liver fractions

Data are from three separate liver samples. The concentration of TFEC was 20 mM.

	Specific activity (munits/mg of protein)	
	AGAT	TFEC β-lyase
Homogenate (H)	25 ± 4	10.1 ± 1.9
Supernatant (S)	15 ± 1	7.1 ± 0.3
Fraction (C1)	10 ± 3	6.5 ± 0.9
Fraction (C2)	23 ± 6	8.1 ± 1.0
Mitochondria (M)	98 ± 8	28.4 ± 0.4
Mitochondria (M)*	50 ± 3†	26.8 ± 1.2

* AGAT II was immunoprecipitated from 50 μl of the mitochondrial homogenate (containing 2.5 mg of protein and about 250 munits of enzyme) with 62.5 μl of antiserum and 12.5 mg of Protein A–agarose as described in the Experimental section. AGAT and TFEC β-lyase activities were measured in the supernatant.

† Different from activity in the untreated mitochondrial fraction with $P = 0.05$

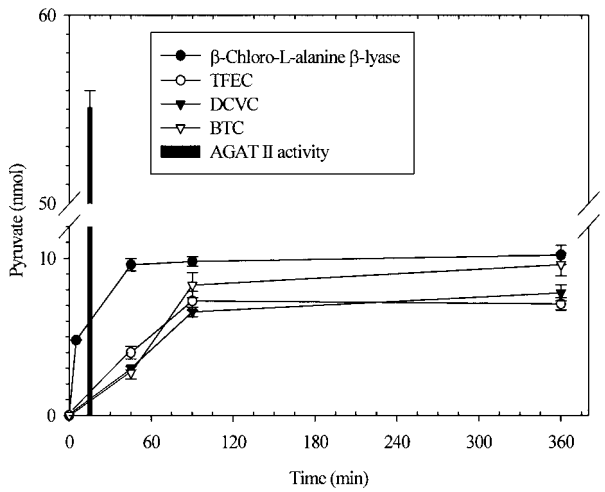

Figure 3 Pyruvate formation catalysed by ipptn. AGAT II with different β-lyase substrates

A suspension of 840 munits of AGAT II immunoadsorbed from a rat kidney mitochondrial homogenate to Protein A-agarose in 1 ml of 10 mM potassium phosphate buffer, pH 7.4, was used in each experiment. The results show the amount of pyruvate formed/10 μl of Protein A–agarose–AGAT II suspension at various time points with different β-lyase substrates (in 100 mM potassium phosphate buffer, pH 8.0). Pyruvate was measured as described in the Experimental section. The concentrations of β-chloro-L-alanine, TFEC, DCVC and BTC were 20, 20, 5 and 5 mM respectively. In each case the blank contained 'Control Protein A' (see the Experimental section). For comparison, the amount of pyruvate formed from a 10 μl aliquot of Protein A–agarose–AGAT II suspension in a standard AGAT reaction mixture at 15 min is shown by the black vertical bar. In a control experiment it was shown that incubation of the Protein A–agarose–AGAT II suspension in 100 mM potassium phosphate buffer, pH 8.0, for 90 min or 3 h at 37 °C resulted in less than 5 % loss of ability of the suspension to catalyse pyruvate formation from the standard AGAT reaction mixture (results not shown). Significantly less pyruvate was formed at 6 h from DCVC and TFEC than from β-chloro-L-alanine ($P = 0.05$).

homogeneous enzyme has a specific activity (aminotransferase assay) of 50 units/mg (i.e. 40/0.8, where 40 is the specific activity of our preparation and 0.8 is the estimated fractional purity). The enzyme suspension (10 μl) used in the experiments depicted in Figure 2 contains 8.4 munits of enzyme or 168 ng of enzyme (assuming that the specific activity of pure enzyme is 50 units/mg). Because the M_r of each subunit is ≈53000 [30], the amount of absorbed enzyme monomer used in each experiment was 3.16 pmol. Thus the ratio of pmol of pyruvate formed per pmol of immobilized monomer inactivated (from the 6 h data) for each of the β-lyase substrates shown in Figure 2 is as follows: β-chloro-L-alanine, 3160 ± 130; TFEC, 2250 ± 130; DCVC, 2470 ± 160; and BTC, 3070 ± 220. Significantly less pyruvate was formed after inactivation with TFEC and DCVC than with β-chloro-L-alanine (Figure 2). The number of turnovers per monomer inactivated was less in the purified (unbound) form than in the form bound to antibody.

AGAT and TFEC β-lyase activities of rat liver

Table 3 shows the relative distribution of AGAT and TFEC β-lyase activities in the fractionated rat liver. The TFEC β-lyase specific activity in the purified rat liver mitochondria (Table 3) is comparable with that of the crude rat kidney mitochondria (Table 1). On the other hand, the TFEC β-lyase specific activity is lower in the cytosolic fractions of rat liver (S, C1, C2; Table 3) than in the cytosolic fraction of rat kidney (Table 1). The lower TFEC β-lyase specific activity in rat liver cytosol than in rat kidney cytosol is presumably a reflection of GTK levels in the two tissues. GTK is a major cysteine S-conjugate β-lyase of rat kidney cytosol under *in vitro* conditions. The activity is considerably less in rat liver cytosol than in rat kidney cytosol [35]. The AGAT specific activity is lower in the highly purified rat liver mitochondria (Table 3) than in the crude rat kidney mitochondria (Table 1). Moreover, unlike the AGAT activity of rat kidney mitochondria, which is almost completely (99 %) precipitated by the AGAT II immune serum (Figure 1), only about 50 % of the activity is precipitated from the rat liver mitochondria (Table 3).

Kinetics of the β-lyase reactions catalysed by purified rat kidney AGAT II

Owing to competing transamination reactions, some aminotransferases that catalyse cysteine S-conjugate β-lyase reactions require the simultaneous presence of a 2-oxo acid substrate or PLP for maximal pyruvate formation via the β-lyase reaction (see the Discussion). To ascertain whether transamination competes with the β-lyase reaction at the active site of AGAT II when TFEC is used as a β-lyase substrate, an attempt was made to measure the amount of L-alanine generated relative to pyruvate (see the Experimental section). The amount of pyruvate formed in the 10 μl/6 h TFEC/AGAT II reaction mixture was 8.9 nmol (or 89 nmol/0.1 ml of reaction mixture). The maximum amount of

Table 4 Kinetic constants for aminotransferase and β-lyase substrates of purified rat kidney AGAT II

The data are the averages of three separate determinations. In all cases the S.E.M. was $< 10\%$ of he mean. The relative V_{max}/K_m value in square brackets for L-alanine is an arbitrary value with which other values are compared.

	Apparent K_m (mM)	V_{max} (μmol/min per mg)	Relative V_{max}/K_m
Transaminase substrate			
L-Alanine	8	56	[1]
β-Lyase substrates			
β-Chloro-L-alanine	0.10	8.0	11
TFEC	10	2.8	0.04
BTC	8	2.0	0.04

L-alanine formed per 0.07 ml of reaction mixture was 1.25 nmol (see the Experimental section). Therefore $\leqslant$1.8 nmol of L-alanine (i.e. 1.25/0.7) is formed for every 89 nmol of pyruvate formed. Thus, with TFEC, the β-elimination reaction catalysed by purified AGAT II is favoured over the transamination reaction by at least a factor of 50. Similar results were found for β-chloro-L-alanine (results not shown). Thus β-lyase activity is not significantly dependent on the presence of added 2-oxo acid substrate. Kinetic parameters for the β-lyase substrates were therefore determined in the absence of added pyruvate, but in the presence pf 10 μM PLP.

The kinetic data shown in Table 4 demonstrate that β-chloro-L-alanine binds more effectively to the active site of AGAT II than does the natural amino acid substrate L-alanine. Maximal pyruvate formation from β-chloro-L-alanine catalysed by AGAT II via the β-lyase reaction is about 14 % the maximal rate of pyruvate formation catalysed by AGAT II via the aminotransferase reaction. As a result the V_{max}/K_m value for β-chloro-L-alanine (as a β-lyase substrate) is 11 times greater than that for L-alanine (as an aminotransferase substrate). In contrast, V_{max} values for pyruvate formation from the β-lyase substrates TFEC and BTC are 4–5 % of the maximal rates of pyruvate formation from transamination of L-alanine. However, the K_m values exhibited by AGAT II toward TFEC and BTC are similar to that exhibited toward L-alanine (Table 4). [A similar analysis could not be carried out with DCVC, because this amino acid absorbs strongly at the wavelength (220 nm) used to monitor pyruvate formation.]

DISCUSSION

Interaction of AGAT II with β-chloro-L-alanine

Aminotransferases, such as AlaAT, cytosolic aspartate aminotransferase (cytAspAT) and mitAspAT, have been reported to catalyse β-lyase reactions with amino acids that contain a good leaving group attached at the β-carbon. For example, β-chloro-L-alanine is a β-lyase substrate of pig heart AlaAT [37] and both isoenzymes of pig heart AspAT [37]. We recently showed that β-chloro-L-alanine is a β-lyase substrate of rat liver mitAspAT [26] and of both isoenzymes of human branched-chain aminotransferase ($BCAT_c$ and $BCAT_m$) [27]. Here, we have confirmed the previous finding of Porter et al. [33] that rat AGAT II possesses considerable β-lyase activity toward β-chloro-L-alanine and is also inactivated by this amino acid. Porter et al. [33] reported a $t_{1/2}$ for inactivation of 4.4 min in the presence of 25 mM β-chloro-L-alanine and potassium phosphate buffer, pH 8.5, a K_m value of 0.07 mM, a β-lyase rate relative to the rate of transamination between L-alanine and glyoxylate of $\approx$23 % and a ratio of pyruvate formed to monomer inactivated of 900. Our corresponding values (phosphate buffer, pH 8.0) are 4.0 min (Table 2), 0.1 mM (Table 4), $\approx$14–20 % (Tables 1 and 4) and 1690 (Table 2) respectively. Our values are reasonably close to those obtained previously by Porter et al. [33].

The K_m values exhibited toward β-chloro-L-alanine by various aminotransferases vary markedly. The values reported for mitAspAT [38], cytAspAT [38], AlaAT [37], $BCAT_m$ (27), AGAT II ([33]; the present study) are 50, 200, 0.1, 0.6, 0.07–0.1 mM respectively. Evidently β-chloro-L-alanine binds favourably at the active sites of some aminotransferases (including AGAT II), but not others.

Binding of amino acids at the active site of AGAT II

Table 5 compares several K_m and V_{max} values for aminotransferase and β-lyase substrates of highly purified rat AGAT II. Our data show that TFEC and BTC bind to the active site of AGAT II (as β-lyase substrates) about as effectively as the various methylated arginines (as aminotransferase substrates), but more effectively than L-α-aminobutyrate and L-ornithine (as aminotransferase substrates). The data also show that the V_{max}/K_m ratios for TFEC and BTC (as β-lyase substrates) are similar to those exhibited toward the methylated arginines (as aminotransferase substrates).

Comments on the ability of AGAT II to catalyse cysteine S-conjugate β-lyase reactions

Seven mammalian aminotransferases {GTK/kynurenine aminotransferase I, cytAspAT, mitAspAT, AlaAT, $BCAT_c$, $BCAT_m$ and AGAT II ([20] and references cited therein) and kynureninase [39]} are currently known to catalyse β-lyase reactions with toxic halogenated cysteine S-conjugates. Because the ability to catalyse β-lyase reactions appears to be a common feature among many aminotransferases, more aminotransferases may eventually be shown to catalyse a cysteine S-conjugate β-lyase reaction. Apparently, the electron-withdrawing properties at (and including the sulphur) are such that, once the halogenated cysteine S-conjugate binds at the active site and reacts with the PLP cofactor to form the quinonoid intermediate, electron flow towards the sulphur results in a non-physiological β-elimination reaction [40]. β-Elimination results in regeneration of the PLP form of the enzyme, which is free to catalyse another round of β-elimination. However, if transamination of the cysteine S-conjugate with the cofactor occurs, the PMP (pyridoxamine 5′-phosphate) form of the enzyme is generated. The PMP form can no longer support a further round of β-elimination. In that case, PLP or 2-oxo acid substrate is required to maintain the β-elimination reaction. In the case of GTK, transamination competes effectively with β-elimination when DCVC is the substrate. As a result, GTK-catalysed β-elimination from DCVC is strongly dependent on added 2-oxo acid substrate [40]. On the other hand, the β-elimination reaction with TFEC catalysed by mitAspAT is only weakly dependent on added 2-oxo acid [26], and the β-elimination reaction with TFEC catalysed by $BCAT_c$ and $BCAT_m$ is not dependent on added 2-oxo acid substrate [27]. In the present work no evidence could be found that transamination competes effectively with the β-lyase reaction when TFEC binds at the active site of AGAT II. The reason for the more effective channelling of electrons toward the sulphur of the halogenated cysteine S-conjugates in AGAT II and the BCAT isoenzymes than in GTK must await detailed comparative studies of the respective active sites.

Table 5 K_m and V_{max} values of aminotransferase and β-lyase substrates of purified rat kidney AGAT II

The relative V_{max}/K_m value in square brackets for L-alanine is an arbitrary value with which other values are compared.

	K_m (mM)	V_{max} (μmol/min per mg)	Relative V_{max}/K_m	Reference
Aminotransferase substrates*				
L-Alanine (G)	11	160	[100]	[29]
	3.2	29.7†	64	[33]
	7.8	31.7	28	[31]
	8.0	56	48	The present study
L-α-Aminobutyrate (G)	60	>76	>9	[29]
L-Ornithine (P)	70	7.5	0.7	[31]
N^G,N'^G-DimethyL-L-arginine (P)	9.7	3.9	2.8	[31]
	13.7	5.2	2.7	[31]
N'^G-MonomethyL-L-arginine (P)	8.0	1.52	1.3	[31]
β-Lyase substrates				
β-Chloro-L-alanine	0.07	6.8†	674	[33]
	0.1	8.0	554	The present study
TFEC	10	2.8	1.9	The present study
BTC	8.0	2.0	1.7	The present study

* Co-substrates for the transamination reaction were glyoxylate (G) or pyruvate (P). The K_m and V_{max} values for transamination of L-alanine and L-α-aminobutyrate reported in [39] and [43] are *absolute* values calculated from Ping Pong plots. The remaining K_m and V_{max} data in the 'Aminotransferase substrate' column are *apparent* values calculated at a fixed (non-infinite) concentration of glyoxylate and pyruvate. Although the methods used by the different authors varied slightly, all determinations were conducted at 37 °C and in phosphate buffer, pH 8.0–8.5.

† Calculated from the catalytic-centre activities ('turnover numbers') reported by the authors.

The V_{max} value exhibited by rat AGAT II toward TFEC as a β-lyase substrate is $\approx$5% that exhibited by L-alanine as an aminotransferase substrate (Tables 4 and 5). Previous estimates of maximal cysteine S-conjugate β-lyase activity versus maximal transamination rates for natural amino acid substrates range from $<$1% for AlaAT to about 15% for $BCAT_m$ and about 40% for GTK (discussed in [20]). However, the V_{max} value for TFEC β-lyase activity exhibited by AGAT II (2.8 μmol/min per mg; Table 4) is of the same order of magnitude as the maximal value reported for DCVC β-lyase activity catalysed by GTK (6.4 μmol/min per mg) [41]. (Because the subunit M_r values of GTK and AGAT II are $\approx$48000 [41] and $\approx$53000 [30] respectively, the ratio of maximal TFEC lyase activity per AGAT II subunit/maximal DCVC lyase activity per GTK subunit is 0.55.)

In each of the aminotransferases listed above, except rat kidney GTK, turnover of the β-lyase substrate leads to inactivation. The ratio of catalytic-centre activity ('turnover number') to monomer inactivated ranges from thousands in the case of rat mitAspAT [26] to about 30 in the case of human $BCAT_c$ [27]. The present work and that of Porter et al. [33] suggests that the susceptibility to inactivation of AGAT II by the β-lyase substrates is similar to that exhibited by rat mitAspAT. Originally it was thought that inactivation of cytAspAT by β-lyase substrates is due to attack of aminoacrylate on a susceptible active-site residue [38]. Whereas this mechanism may hold for inactivation of bacterial D-amino acid aminotransferase by β-chloro-D-alanine [42], it does not hold for inactivation of bacterial glutamate decarboxylase or pig heart cytAspAT by L-serine *O*-sulphate [43]. In those cases, inactivation by the β-lyase substrate is due to aminoacrylate reacting with the PLP cofactor, forming a pyruvate–PLP aldol product [43]. Another mechanism of inactivation is possible in the case of TFEC (and DCVC), but not with β-chloro-L-alanine or BTC. In the case of TFEC (and DCVC) the eliminated thioacylating fragment may attack a crucial active-site residue. In this context it is noteworthy that, after rats were administered TFEC, thioacylation of kidney mitAspAT was detected [21]. Determination of whether TFEC-induced inactivation of AGAT II is due to PLP-aldol formation, aminoacrylate attack on an active-site residue, thioacylation of an active-site residue or a combination of two or more mechanisms, must await further study. However, it is worth noting that TFEC is significantly more effective at inactivating AGAT II (i.e. lower ratio of turnovers to inactivation) than is β-chloro-L-alanine, suggesting that there may be subtle differences in the mode of inactivation between the two β-lyase substrates. It is also of interest that the immunoprecipitated enzyme appears to be more resistant to inactivation. Possibly the susceptible groups/residues may be more shielded in the immunoprecipitated enzyme.

Possible contribution of AGAT II to kidney and liver damage induced by halogenated cysteine S-conjugates

Mammalian tissues contain at least three aminotransferases that utilize L-alanine as a major amine donor. AlaAT utilizes 2-oxoglutarate as amine acceptor and is most active in liver cytosol. This enzyme has only weak cysteine S-conjugate β-lyase activity ([20] and references cited therein) and alanine-2-oxoglutarate aminotransferase activity is low in rat kidney [44]. For these reasons, AlaAT is unlikely to be a major contributor to the bioactivation of toxic halogenated cysteine S-conjugates in rat kidney mitochondria. AGAT I and AGAT II also preferentially utilize L-alanine as amine donor. However, unlike AlaAT, the amine acceptor is not 2-oxoglutarate, but rather glyoxylate. AGAT I is a class IV aminotransferase that is localized in peroxisomes, mitochondria, or both, depending on the species [45,46]. Its variable subcellular location in different species is possibly due to the influence of dietary selection pressure [46]. In most humans, AGAT I is peroxisomal, but, in a subset of patients with hyperoxaluria type 1, the functional enzyme is mistargeted to mitochondria [47]. In rat liver, AGAT I is distributed about equally between peroxisomes and mitochondria [28]. In future studies it will be interesting to determine whether AGAT I can catalyse a cysteine S-conjugate β-lyase reaction.

AGAT II is exclusively mitochondrial in mammals [28–30,45]. Rat liver contains both AGAT I and AGAT II, whereas rat kidney contains only AGAT II [28,45]. In accord with this finding,

almost all the AGAT activity was removed from crude rat kidney homogenates with AGAT II immune serum/Protein A–agarose (Figure 2). On the other hand, only about 50 % of the AGAT activity was removed from liver mitochondria by such treatment (Table 3). The remainder of the activity in the liver mitochondria is presumably due to AGAT I. Rat liver fractions C1 and C2 contain peroxisomes that are not pelleted under conditions that pellet mitochondria. Thus AGAT activities in these fractions (Table 3) are most likely due to peroxisomal AGAT I. Our findings show that $\approx$50 % of the AGAT activity of rat liver is due to AGAT II. This finding is consistent with the findings of Porter et al. [33], who showed that the ratio of (*R*,*S*)-fluoro-β-alanine defluorinase specific activity to AGAT specific activity was three times higher in a highly purified rat liver AGAT II preparation than in the homogenate. This finding implies that AGAT II accounts for only about 33 % of the total AGAT activity of rat liver. A major portion of the remaining activity (67 %) is most probably due to AGAT I. A comparison of Tables 1 and 3 shows that the specific activity of AGAT II in rat liver mitochondria is only about 25 % that in rat kidney mitochondria. Our data suggest that AGAT II is a major cysteine S-conjugate of rat kidney, but proportionately less so of rat liver.

We have previously suggested that, because mitAspAT and $BCAT_m$ possess cysteine S-conjugate β-lyase activity, these enzymes may contribute to the toxicity of halogenated cysteine S-conjugates [26,27]. We have also presented evidence that these enzymes may contribute to channeling of toxicants (i.e. the reactive-sulphur-containing fragments) and inactivation of closely juxtaposed key mitochondrial enzymes of energy metabolism [20]. However, because mitAspAT and $BCAT_m$ are widely distributed, there must be additional factors that contribute to the targeting of rat kidney and, to a lesser extent, liver and other organs by toxic halogenated cysteine S-conjugates. Such factors include the ability of the cell to take up the conjugate, the ability of the cell to concentrate the conjugate in the mitochondria, the ability of the cell to acylate and deacylate the conjugate, protection by endogenous substrates and proximity of proteins susceptible to thioacylation. A major factor in the targeting of kidneys by halogenated cysteine S-conjugates (and indeed many drugs) is the low mass of the kidneys ($<$1 % of the total body mass) yet high renal blood flow ($\approx$20 % of the resting cardiac output) coupled with high transport systems and high water recovery (i.e. strong concentrative abilities) [48]. Because (a) AGAT II possesses cysteine S-conjugate β-lyase activity and (b) it is present in rat kidney mitochondria at high levels and to a lesser extent in rat liver mitochondria [28], the enzyme may also contribute to the targeting of these tissues in the rat by toxic halogenated cysteine S-conjugates. Possibly, some of the toxicity may also be due to loss of AGAT II activity if exposure to the toxic cysteine S-conjugates is high. In future work it will be interesting to determine whether AGAT II in rat kidney and liver mitochondria is associated with other mitochondrial enzymes of energy metabolism and can participate in channeling of reactive sulphur-containing fragments to these enzymes.

AGAT II was reported to be absent from human liver and kidney [28]. However, as pointed out in [33], some individuals do actually have this activity. D-3-Aminoisobutyrate aminotransferase is identical with AGAT II [49], and this activity is present in the livers of humans who are low excretors of D-3-aminoisobutyrate, but is absent in the livers of humans who are high excretors [50]. A breakdown product of the widely used anticancer drug 5-fluorouracil is fluoride, and this ion may contribute to the toxicity of the drug ([33] and references cited therein). Porter et al. [33] have presented evidence that fluoride arises via conversion of 5-fluorouracil into (*R*)-α-fluoro-β-alanine, followed by α,β-elimination catalysed by AGAT II. The present findings suggest that AGAT II may also be involved in the mitochondrial bioactivation of halogenated cysteine S-conjugates. The present findings and those of Porter et al. [33] suggest that AGAT II may have a more prominent role in drug/xenobiotic metabolism in humans than previously recognized and that variations in the level of this enzyme may account for differences in the metabolism of some drugs and other xenobiotics. AGAT II has been little studied, but its role in bioactivation processes deserves further study.

Conclusion

In its capacity to catalyse cysteine S-conjugate β-lyase reactions, AGAT II may contribute to the well known targeting of TFEC, DCVC and other toxic halogenated cysteine S-conjugates to mitochondria in kidney and, to a lesser extent, in liver in the rat. The enzyme may also contribute to the bioactivation of toxic halogenated cysteine S-conjugates and other xenobiotics in a subset of human beings.

This work was supported by NIH Grants RO1 ES008421 and PO1 AG14930. We thank Dr Sou-Youl Kim (Burke Medical Research Institute) for performing the SDS/PAGE analysis of purified AGAT II.

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Received 1 July 2003; accepted 15 July 2003
Published as BJ Immediate Publication 15 July 2003, DOI 10.1042/BJ20030988