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Human mitochondrial and cytosolic branched-chain aminotransferases are cysteine *S*-conjugate β-lyases, but turnover leads to inactivation

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Abstract

The mitochondrial and cytosolic branched-chain aminotransferases ($BCAT_m$ and $BCAT_c$) are homodimers in the fold type IV class of pyridoxal 5′-phosphate-containing enzymes that also contains D-amino acid aminotransferase and 4-amino-4-deoxychorismate lyase (a β-lyase). Recombinant human $BCAT_m$ and $BCAT_c$ were shown to have β-lyase activity toward three toxic 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 toward β-chloro-L-alanine. Human $BCAT_m$ is a much more effective β-chloro-L-alanine β-lyase than two aminotransferases (cytosolic and mitochondrial isozymes of aspartate aminotransferase) previously shown to possess this activity. $BCAT_m$, but not $BCAT_c$, also exhibits measurable β-lyase activity toward a relatively bulky cysteine *S*-conjugate [benzothiazolyl-L-cysteine]. Benzothiazolyl-L-cysteine, however, inhibits the L-leucine–α-ketoglutarate transamination reaction catalyzed by both enzymes. Inhibition was more pronounced with $BCAT_m$. In the presence of β-lyase substrates and α-ketoisocaproate (the α-keto acid analogue of leucine), no transamination could be detected. Therefore, with an amino acid containing a good leaving group in the β position, β-elimination is greatly preferred over transamination. Both BCAT isozymes are rapidly inactivated by the β-lyase substrates. The ratio of turnover to inactivation per monomer in the presence of toxic halogenated cysteine *S*-conjugates is ∼170–280 for $BCAT_m$ and ∼40–50 for $BCAT_c$. Mitochondrial enzymes of energy metabolism are especially vulnerable to thioacylation and inactivation by the reactive fragment released from toxic, halogenated cysteine *S*-conjugates such as *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine. The present results suggest that BCAT isozymes may contribute to the mitochondrial toxicity of these compounds by providing thioacylating fragments, but inactivation of the BCAT isozymes might also block essential metabolic pathways.

Keywords: Cytosolic branched-chain aminotransferase; Mitochondrial branched-chain aminotransferase; Cysteine *S*-conjugates; Cysteine *S*-conjugate β-lyases; Halogenated alkenes

1. Introduction

PLP-dependent enzymes have been classified into four families with different fold types. Most aminotransferases, including the well-studied AspAT [1,2], belong to fold type I [3]. On the other hand, $BCAT_m$ and the cytosolic isoform ($BCAT_c$) belong to the fold type IV family of PLP-dependent enzymes [3–5]. Only two other enzymes are currently known to belong to the fold type IV family. These are bacterial DAAT [6–9] and ADCL [10,11]. ADCL catalyzes the conversion of 4-amino-4-deoxychorismate to *p*-aminobenzoate and pyruvate (a β-lyase reaction).

$BCAT_m$ is widely expressed in tissues including kidney and brain (reviewed in Ref. [12]). On the other hand, $BCAT_c$ is present only in nervous tissue, and to a lesser

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Abbreviations: ADCL, 4-amino-4-deoxychorismate lyase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; BCAT, branched-chain aminotransferase (unspecified form); $BCAT_c$, cytosolic branched-chain aminotransferase; $BCAT_m$, mitochondrial branched-chain aminotransferase; BTC, benzothiazolyl-L-cysteine; cyt, cytosolic; CTFC, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; DAAT, D-amino acid aminotransferase; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DTT, dithiothreitol; GDH, glutamate dehydrogenase; GTK, glutamine transaminase K; KIC, α-ketoisocaproate; LDH, lactate dehydrogenase; LeuDH, leucine dehydrogenase; mit, mitochondrial; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; and TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

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extent in ovary and placenta [12]. $BCAT_m$ has a well-defined role in the metabolism of whole-body branched-chain amino acids [13]. The metabolic role of $BCAT_c$ is less clear. The occurrence of both isoforms in nervous tissue, however, suggests a unique aspect to branched-chain amino acid metabolism in brain. Indeed, the enzymes may be important in replenishing the nitrogen of neurotransmitter glutamate, and in nitrogen cycling between astrocytes and neurons [14–23].

It has long been known that aminotransferases such as DAAT [24], cytAspAT [25–27], mitAspAT [26], and AlaAT [28] can catalyze β-lyase reactions with amino acids containing a good leaving group in the β position. Aminoacrylate [$CH_2{=}C(NH_3^+)CO_2^-$] is released from the active site, which then undergoes bond rearrangement and hydrolysis to pyruvate and ammonia. The net reaction (Eq. (1)) is:

$$XCH_2CH(CO_2^-)NH_3^+ + H_2O \rightarrow CH_3C(O)CO_2^- + NH_4^+ + X^- \quad (1)$$

The early studies used β-chloro-L-alanine (or the D-isomer) and L-serine *O*-sulfate [24–28]. DAAT, cytAspAT, mitAspAT, and AlaAT are all inactivated syncatalytically during turnover of the β-lyase substrates [24–28]. More recently, it was shown that cytAspAT [29–33], AlaAT [30,33], and mitAspAT [34] can catalyze β-lyase reactions with cysteine *S*-conjugates containing a good leaving group in the β position (Eq. (1), X = RS). Slow syncatalytic inactivation of purified pig heart cytAspAT, pig heart AlaAT, and rat liver mitAspAT was shown to occur with DCVC and TFEC [33,34].

Several halogenated alkenes (e.g. trichloroethylene, tetrachloroethylene, and tetrafluoroethylene) are heavily used in industry. In the case of trichloroethylene, there is some concern not only for the exposed workers, but for the general population, because this compound is a major environmental contaminant. Trichloroethylene causes renal and liver tumors in experimental animals (e.g. Refs. [35,36]). Although there has been some past debate on the issue, trichloroethylene is almost certainly a human renal carcinogen (e.g. Refs. [37–39]). Tetrafluoroethylene, the precursor of Teflon™, produces both hepatocellular carcinomas and kidney cell adenomas in rodents [40], and chronic inhalation of this haloalkene results in damage to the renal proximal tubules in rats [41]. Lifetime exposure to tetrachloroethylene (perchloroethylene, perc) induces a low level of renal tumors in rats [42]. Halogenated alkenes are metabolized at least in part to the corresponding cysteine *S*-conjugates (DCVC, TFEC, and CTFC are the cysteine *S*-conjugates corresponding to trichloroethylene, tetrafluoroethylene, and chlorotrifluororethylene, respectively.) Much evidence suggests that cysteine *S*-conjugates are a major factor in the nephrotoxicity of halogenated alkenes (e.g. Ref. [43] and references cited therein). Within the kidney, the proximal tubules, especially the S3 region, are especially sensitive. Toxicity of DCVC has been demonstrated in isolated rat (e.g. Ref. [44]) and human [45] kidney proximal tubules, and in cultured human proximal tubule cells [46]. Toxicity is due in part to the high reactivity of the sulfur-containing fragment eliminated by the action of cysteine *S*-conjugate β-lyases. Evidence suggests that the fragments eliminated from DCVC and TFEC (and CTFC) breakdown to a thioketene [47] and a dihalothionoacetyl fluoride [43,48], respectively, both of which act as thioacylating agents particularly of lysine residues in proteins [49–52]. Proteins in the kidney mitochondria are especially vulnerable to thioacylation after rats are administered TFEC. Several mitochondrial enzymes of energy metabolism are inactivated in kidney cells [53–55], PC12 cells [56], and hepatocytes [55] exposed to TFEC. Because of the potential for human exposure to halogenated alkenes in the workplace and in the environment, it is important to characterize the cysteine *S*-conjugate β-lyases that may contribute to the bioactivation of halogenated cysteine *S*-conjugates. (For reviews, see Refs. [57–63].)

Inasmuch as (a) β-lyase activity appears to be a general property of many aminotransferases including DAAT (a fold class IV PLP enzyme), and (b) ADCL (another fold class IV enzyme) naturally catalyzes a β-lyase reaction, the fold class IV BCAT isozymes should, theoretically, also be able to catalyze effective β-lyase reactions. The present work shows that both BCAT isozymes catalyze β-lyase reactions with toxic halogenated cysteine *S*-conjugates and with β-chloro-L-alanine. Turnover was shown to lead to inactivation. The relatively bulky BTC was found to be a β-lyase substrate and inactivator of $BCAT_m$. Inactivation was more pronounced at higher pH values. BTC was neither a β-lyase substrate nor an irreversible inhibitor of $BCAT_c$. On the other hand, BTC inhibited transamination between leucine and α-ketoglutarate catalyzed by both enzymes, but inhibition was somewhat more pronounced with $BCAT_m$.

2. Materials and methods

2.1. Reagents and enzymes

Ammediol (2-amino-2-methyl-1,3-propanediol), Tris, L-leucine, β-chloro-L-alanine·HCl, PLP, DTT, EDTA, 2,4-dinitrophenylhydrazine, NADH, NAD^+, ADP, rabbit muscle LDH (type XXXIX; 720 U/mg of protein in 50% glycerol; 2.9 mg/mL), beef liver GDH (type II; 50 U/mg of protein in 50% glycerol; 10 mg/mL), and the sodium salts of pyruvate, KIC, and α-ketoglutarate were obtained from the Sigma Chemical Co. Bacterial L-LeuDH (38 U/mg of protein; lyophilized powder) was obtained from the Toyobo Co., Ltd. TFEC was synthesized as described previously [49]. DCVC and CTFC were gifts from Dr. James L. Stevens (Lilly Research Laboratories). Stock solutions of potential β-lyase substrates used in the present

experiments, namely 20 mM BTC (acetate salt; synthesized as described [64]) in 100 mM ammediol–HCl buffer (pH 9.0), 40 mM TFEC in 100 mM Tris–HCl buffer (pH 8.0), 40 mM CTFC in 100 mM Tris–HCl buffer (pH 8.0), 20 mM DCVC in 100 mM Tris–HCl buffer (pH 8.0), and 100 mM β-chloro-L-alanine·HCl (neutralized with NaOH) in distilled water, were stored at −20°. In some cases, the pH of the BTC solution was adjusted to pH 7.6–8.0 with 1 M HCl before use. Recombinant human $BCAT_c$ and $BCAT_m$ were overexpressed in *Escherichia coli* using a pET-28a vector carrying a sequence encoding an N-terminal His·Tag/thrombin/T7·Tag. The His·Tag was removed and the enzymes were purified as described [65,66]. $BCAT_m$ and $BCAT_c$ were stored at −20° in a solution containing 150 mM NaCl, 5 mM DTT, 1 mM EDTA, 1 mM KIC, 1 mM glucose, and 10% (v/v) glycerol in 25 mM Tris–HCl (pH 7.5). Each BCAT preparation was subjected to mass spectrometry and shown to be >99% pure. A unit of BCAT activity is the amount of enzyme that catalyzes the formation of 1 μmol/min of L-[1-^{14}C]valine at 37° in a standard reaction mixture containing 1 mM α-ketoiso[1-^{14}C]valerate and 12 mM L-isoleucine [65,66].

2.2. Measurement of BCAT activity

BCAT activity was measured by a new 96-well plate spectrophotometric procedure [67]. The standard reaction mixture (0.2 mL) contained 5 μM PLP, 50 mM ammonium sulfate, 0.05 mM NADH, 5 mM DTT, 5 mM α-ketoglutarate, 10 mM L-leucine, and 0.95 U of LeuDH in 100 mM potassium phosphate buffer (pH 7.4)[1]. The reaction was initiated by the addition of BCAT. The disappearance of absorbance at 340 nm (ε 6230 M^{-1} cm^{-1}) was measured continuously at 37°. Under the conditions of the spectrophotometric assay, the specific activities of the purified BCATs from two different $BCAT_m$ preparations and two different $BCAT_c$ preparations were 64, 70, 86, and 110 U/mg of protein, respectively. The published values determined using the radioisotope assay are: 88 ± 6 U/mg of $BCAT_m$ and 124 ± 9 U/mg of $BCAT_c$ [65,66].

2.3. Measurement of products obtained in various enzyme reactions

In most cases, pyruvate formed in the presence of β-lyase substrates and the BCATs was measured as its 2,4-dinitrophenylhydrazone in an end-point assay. Twenty microliters of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added to a 20-μL solution containing pyruvate. After incubation at 37° for 10 min, 160 μL of 1 M NaOH was added, and the absorbance of the pyruvate 2,4-dinitrophenylhydrazone at 430 nm was determined within 2 min in a SpectraMax 96-well plate analyzer against a blank consisting of 20 μL of water (plus enzyme) carried through the same procedure. The extinction coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions is 15,000 M^{-1} cm^{-1}. In some cases, pyruvate was measured enzymatically with LDH either in an end-point assay or continuously. Although LDH has a broad specificity toward many α-keto acids, its activity toward KIC is negligible (Ref. [68] and verified here). Therefore, it is possible to measure pyruvate with LDH even in the presence of a large excess of KIC. In the end-point LDH assay, 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 SpectraMax 96-well plate analyzer. The conversion of pyruvate to lactate is complete within about 2 min. In the continuous LDH assay, pyruvate generated from β-lyase substrates was monitored over time in a 0.2-mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.4), 7.5 μg LDH, 0.05 mM NADH, and β-lyase substrate by measuring the rate of decrease in absorbance at 340 nm.

Ammonia was measured enzymatically 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 α-ketoglutarate, and 100 mM potassium phosphate buffer (pH 7.4), and the decrease in absorbance at 340 nm was monitored continuously. The conversion of ammonia to L-glutamate is complete in 10 min at 37° at which time the slow drift in absorbance loss at 340 nm in the sample well equals that in the blank (total reaction mixture lacking ammonia).

L-Leucine was measured enzymatically with LeuDH. The procedure relies on the fact that the oxidative deamination of L-leucine is favored at high pH values (≥10) and that LeuDH is catalytically competent at these high pH values. To a 20-μL solution containing L-leucine was added 180 μL of a reaction mixture containing 4 mM NAD^+, 25 μg LeuDH, 100 mM sodium carbonate/bicarbonate buffer (pH 10.4). The increase in absorbance at 340 nm due to reduction of NAD^+ was determined. The oxidation of L-leucine/reduction of NAD^+ is complete in about 5 min at 37°. The oxidation of L-leucine is complete even in the presence of millimolar amounts of KIC.

For experiments in which reactions were carried out in small volumes (typically 20 μL) for up to 1 hr, small snap-top tubes were used to prevent excessive evaporation. The samples were then transferred quantitatively to a well plate for analyses of pyruvate, ammonia, or leucine.

The limit of detection for pyruvate in the 2,4-dinitrophenylhydrazone assay is 0.5 nmol ($\Delta OD_{430\,nm} = 0.019$). Limits of detection for pyruvate and ammonia coupled to NADH disappearance, and leucine coupled to NADH appearance, are about 1 nmol ($\Delta OD_{340\,nm} = 0.016$).

[1] The PLP concentration was kept low because this compound can catalyze some non-enzymatic formation of pyruvate when incubated with β-chloro-L-alanine, TFEC, DCVC, or CTFC. The non-enzymatic formation of pyruvate from these amino acids in the presence of 5 μM PLP was about 1–5 nmol/hr/20 μL reaction mixture at 37°.

Table 1
Pyruvate formed from β-lyase substrates with $BCAT_m$ in the presence of 0.25 mM KIC[a]

L-Amino acid	Concentration (mM)	Pyruvate formed (nmol)		% Activity at 1 hr[b]
		Incubation time		
		2.5 min	1 hr	
None				95, 98
BTC[c]	5	5.2 ± 1.2	40.0 ± 3.2	73, 78
BTC[d]	5	4.4 ± 1.2[e]	23.3 ± 2.1	3, 6
TFEC	10	4.7 ± 0.1	22.0 ± 1.6	12, 18
DCVC	5	2.5 ± 0.5	14.3 ± 2.6	40, 44
CTFC	10	2.5 ± 0.2	28.5 ± 0.7	5, 15
β-Chloro-L-alanine	25	38.0 ± 0.3[e]	43.2 ± 0.3	<1, <1

[a] $BCAT_m$ (6.25 μg, 0.4 U) was incubated at 37° in a 20-μL reaction mixture containing 5 μM PLP, 100 mM potassium phosphate buffer (pH 7.4), and the amino acids indicated. Due to carryover from the $BCAT_m$ storage buffer, the concentration of KIC in the reaction mixture was 0.25 mM. At the times indicated, pyruvate was measured as its 2,4-dinitrophenylhydrazone. The blanks consisted of incubation mixture incubated for 2.5 or 60 min followed by the addition of enzyme and 2,4-dinitrophenylhydrazine reagent. No loss of activity was noted when enzyme was incubated for 1 hr at 37° in any of the buffer systems in the absence of β-lyase substrate. The data for pyruvate formation are expressed as means ± SEM (N = 3).

[b] In a separate experiment, an aliquot of the reaction mixture was removed at 1 hr and assayed for aminotransferase activity (N = 2).

[c] This experiment was carried out separately from the experiment in which the remainder of the data was collected. Relative activities in the control (no addition) were 93 and 100% at 1 hr.

[d] In this experiment, the pH of the stock BTC solution was not adjusted with HCl so that the final pH of the solution was 9.0.

[e] In a separate experiment, the amount of pyruvate formed at 2.5 min as determined with the end-point LDH procedure was 35 and 5.2 nmol for the β-lyase reaction catalyzed by $BCAT_m$ in the presence of 25 mM β-chloro-L-alanine and 5 mM BTC, respectively.

2.4. Statistical analyses

For determinations where N was ≥3, the mean ± SEM is reported. Statistical comparisons were carried out using the Mann–Whitney *U* test; $P \leq 0.05$ was considered significant.

3. Results

3.1. Demonstration that $BCAT_m$ has cysteine S-conjugate β-lyase activity

Table 1 shows that $BCAT_m$ has β-lyase activity toward cysteine *S*-conjugates and toward β-chloro-L-alanine. In most of the determinations, pyruvate was measured as its 2,4-dinitrophenylhydrazone. The assay was validated in two separate experiments by measuring pyruvate formation with LDH. As shown in Table 1, the LDH and 2,4-dinitrophenylhydrazone methods gave comparable results. Table 1 also shows that the enzyme was concomitantly inactivated by the β-lyase substrates. In the case of BTC, inactivation was more pronounced at pH 9.0 than at pH 7.4, but β-lyase activity was more pronounced at the lower pH.

3.2. Stoichiometry of pyruvate, ammonia, and leucine formation

The incubation mixture used in the experiment described in Table 1 contained 0.25 mM KIC (carryover from the $BCAT_m$ storage buffer) and 5 μM PLP. Both of these compounds will maintain $BCAT_m$ in the PLP form should transamination occur. To detect possible $BCAT_m$-catalyzed transamination between β-chloro-L-alanine (or cysteine *S*-conjugates) and KIC, the concentration of KIC was increased from 0.25 to 10 mM. The reaction mixtures were incubated for 1 hr at 37° and then analyzed for pyruvate, ammonia, and L-leucine[2] as described in "Section 2" (Table 2).

Table 2 shows that the amount of ammonia generated in the $BCAT_m$-catalyzed β-lyase reaction with β-chloro-L-alanine, BTC, and TFEC in the presence of KIC was similar to the amount of pyruvate generated. Within the limits of the sensitivity of the assay (1 nmol), no L-leucine could be detected. To ensure that the assay system for leucine was not compromised in the presence of the halogenated amino acids, 20 nmol of L-leucine was added to each assay mixture. In every case, the appearance of absorbance at 340 nm was rapid, reaching a maximum within 5 min. The results show that $BCAT_m$-catalyzed transamination of β-chloro-L-alanine, BTC, and TFEC is negligible, i.e. β-elimination is greatly favored over transamination.

3.3. Inactivation of $BCAT_m$ by β-lyase substrates at 37°

Comparison of pyruvate formation at 2.5 and 60 min indicated that the enzyme is inactivated during turnover of β-lyase substrates (Table 1), and this was shown directly (last column of Table 1). The time course for the inactivation of the enzyme by 5 mM BTC at 37° (pH 9.0) is given

[2] In the blank reaction mixture containing TFEC, inactivated $BCAT_m$, and active LeuDH, a slow appearance of NADH was noted. The change in absorbance at 340 nm over 20 min in the blank containing 10 mM TFEC was about 0.120. Apparently, TFEC is a weak substrate of LeuDH (~15 nmol/min/mg). This value is about 0.03%, the rate observed with 10 mM L-leucine.

Table 2
Pyruvate, ammonia, and L-leucine formed after a 1-hr incubation in reaction mixtures containing β-lyase substrates and a high concentration of KIC[a]

β-Lyase substrate	Concentration (mM)	Product formation (N = 3) (nmol)			$BCAT_m$ activity remaining (%) (N = 2)
		Pyruvate	Ammonia	L-Leucine	
TFEC	10	9.6 ± 0.3	7.1 ± 0.4	<1[b]	47, 59
β-Chloro-L-alanine	25	25.2 ± 3.3	31.0 ± 5.2	<1	18, 22

[a] Reaction mixtures (20 μL) containing $BCAT_m$ (6.25 μg, 0.4 U), 100 mM potassium phosphate buffer (pH 7.4), 5 μM PLP, 10 mM KIC, and the amino acids indicated were incubated for 1 hr at 37°. Individual mixtures (20 μL) were then analyzed separately for pyruvate (by the end-point LDH method), ammonia, and L-leucine as described in "Section 2." In a parallel experiment, 2-μL aliquots were removed after the 1-hr incubation and assayed for $BCAT_m$ activity using the α-ketoglutarate–L-leucine transamination assay. The data for the pyruvate and ammonia concentrations are expressed as means ± SEM.
[b] Corrected for slow appearance of NADH in the blank lacking $BCAT_m$.

in more detail in Fig. 1. The $T_{1/2}$ for inactivation was about 6 min. The enzyme was inactivated more readily by BTC at pH 9.0 than at pH 7.4. Thus, the enzyme was >90 and ~25% inactivated at pH 9.0 and 7.4, respectively, at 1 hr (Table 1). Analysis of enzyme activity after a 1-hr incubation with BTC (pH 7.4) by the radiochemical assay showed 15–20% inactivation (data not shown). A similar experiment showed that the $T_{1/2}$ for inactivation by 25 mM β-chloro-L-alanine at 37° (pH 7.4) is extremely rapid (<1 min). To obtain a more accurate estimate, pyruvate formation was monitored continuously with LDH and NADH (see "Section 2"). When 71.4 ng (5 mU) of $BCAT_m$ was used, the rate of loss of NADH absorbance at 340 nm declined exponentially with a $T_{1/2}$ of 40 ± 3 sec (N = 3) at 37°.

3.4. Protection by KIC against β-chloro-L-alanine- and TFEC-induced inactivation of $BCAT_m$

A comparison of Tables 1 and 2 shows that the amount of pyruvate formed from TFEC and β-chloro-L-alanine in 1 hr when the incubation mixture contained 10 mM KIC was less than that formed when the reaction mixture contained 0.25 mM KIC. Additionally, incubation of $BCAT_m$ with 25 mM β-chloro-L-alanine for 30 min in the presence of 0.25 mM KIC resulted in >99% inactivation (data not shown). Analysis of the reaction mixtures containing 10 mM KIC for residual $BCAT_m$ activity after a 1-hr incubation showed the enzyme to be about 80% inactivated when co-incubated with 25 mM β-chloro-L-alanine (Table 2). The lower extent of inactivation in the presence of 10 mM KIC than in the presence of 0.25 mM KIC shows that high concentrations of KIC partially protect against inactivation by β-lyase substrates.

3.5. Calculation of turnover to inactivation ratios

Human recombinant $BCAT_m$ is a homodimer with one catalytic site per monomer (M_r of the subunit is 41,730 [65]). In a reaction mixture containing 0.150 nmol of $BCAT_m$ subunits and 5 mM BTC, the enzyme was 95% inactivated at pH 9.0 when ~24 nmol of pyruvate had been formed (Fig. 1). Under these conditions, the results show

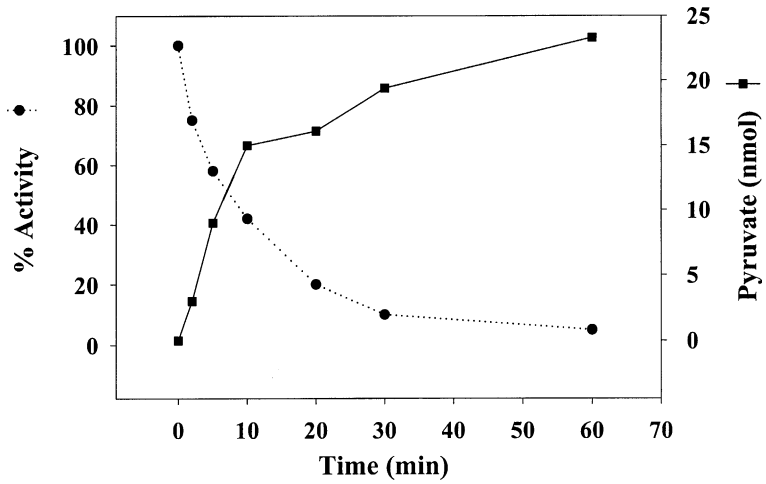

Fig. 1. Inactivation of $BCAT_m$ by BTC. The reaction mixture (1.0 mL) containing 200 mM potassium phosphate, 5 mM DTT, 5 μM PLP, 5 mM BTC, and 6.25 μg (0.4 U) of $BCAT_m$ was incubated at 37° in a 1.5-mL snap-top tube. Due to ammediol buffer in the BTC stock solution, the final pH of the mixture was 9.0. At intervals, 2-μL aliquots were withdrawn and assayed for $BCAT_m$ activity. At the same time, 10-μL aliquots were withdrawn and assayed for pyruvate formation by the 2,4-dinitrophenylhydrazine procedure. In a control experiment, no loss of $BCAT_m$ activity was found when the enzyme was incubated for 1 hr under identical conditions except that BTC was replaced with 25 mM ammediol buffer. The data points are the average of two determinations. The difference between the two values was <10% for all duplicate measurements.

that on average one enzyme monomer turns over about 170 molecules of BTC to pyruvate before inactivation occurs. However, the data in Table 1 suggest that at pH 7.4 the turnover to inactivation ratio is much higher (~1000). The mechanism for the greater susceptibility to inactivation by BTC at higher pH values is not yet clear. The results in Table 1 show that the maximal amount of pyruvate formed from β-chloro-L-alanine was about 43.2 nmol, which suggests that on average ~280 molecules of pyruvate are formed from β-chloro-L-alanine for every molecule of enzyme monomer inactivated. The data in Table 1 suggest that for TFEC, DCVC, and CTFC ~200 molecules are turned over for every enzyme monomer inactivated.

3.6. Interaction of $BCAT_m$ with β-lyase substrates at 23°

As noted above, inactivation of $BCAT_m$ by β-chloro-L-alanine is rapid at 37°. To slow the rates of inactivation so that apparent K_m values could be determined for the β-lyase substrates under initial rate conditions, kinetic constants were determined at 23° (Table 3). The enzyme was found to exhibit a higher affinity and higher V_{max} toward β-chloro-L-alanine than toward TFEC. In accord with the more effective binding of β-chloro-L-alanine to the active site, this amino acid is a better inhibitor of the $BCAT_m$-catalyzed L-leucine–α-ketoglutarate transamination reaction than is TFEC (Table 4).

3.7. Demonstration that $BCAT_c$ has β-lyase activity

Table 5 shows that human $BCAT_c$ can also catalyze the formation of pyruvate from several cysteine *S*-conjugates and β-chloro-L-alanine. In this respect, the enzyme is similar to its mitochondrial counterpart, but there are some notable differences. For example, it is more rapidly inactivated by the β-lyase substrates. Under the conditions shown in Table 5, the enzyme was inactivated ≥95% by β-chloro-L-alanine, TFEC, DCVC, and CTFC at 5 min compared to the control (enzyme incubated in the absence of β-lyase substrate) (data not shown). Moreover, BTC was not a measurable substrate. Under the conditions shown in Table 5, after a 1-hr incubation at 37°, no $BCAT_c$-catalyzed pyruvate formation from BTC (5 mM) could be detected (Table 1). Moreover, little or no loss of activity (0–10%) was noted either by the coupled enzyme assay or by the radiochemical assay after the 1-hr incubation at pH 7.4. Even after incubation at pH 9.0 for 1 hr in the presence of 5 mM BTC, little or no inactivation occurred (≤10%) (data not shown).

$BCAT_c$ is a homodimer (M_r of the recombinant human $BCAT_c$ subunit is ~43,400) [65]. With β-chloro-L-alanine, TFEC, DCVC, and CTFC, on average about 5–6 nmol of pyruvate was formed in the presence of 0.115 nmol of $BCAT_c$ monomers before complete inactivation occurred

Table 3
Kinetic constants exhibited by $BCAT_m$ toward β-lyase substrates at 23° [a]

Substrate	Apparent K_m (mM)	V_{max} (nmol/min/μg)
TFEC	8.4	0.3
β-Chloro-L-alanine	0.6	5.1

[a] The reaction mixture (0.2 mL) contained varied amounts of β-lyase substrate, 100 mM potassium phosphate buffer (pH 7.4), $BCAT_m$ (1.8 μg, 126 mU), 7.5 μg LDH, and 0.05 mM NADH. The rate of loss of absorbance was measured at 340 nm. The pH of the stock solutions of TFEC was adjusted to ~pH 7.4 with 1 M HCl before addition to the reaction mixtures. K_m and V_{max} values were obtained from Lineweaver–Burk plots of 1/v versus 1/[S] and are the averages of three separate determinations. The specific activity of $BCAT_m$ in the standard leucine–α-ketoglutarate aminotransferase assay at 23° was 21 nmol/min/μg.

Table 4
$BCAT_m$-catalyzed transamination of L-leucine with α-ketoglutarate in the presence of β-lyase substrates or L-alanine [a]

Addition	% Relative activity
None	[100 ± 5]
β-Chloro-L-alanine (10 mM)	15 ± 2 [b]
TFEC (4 mM)	85 ± 2 [b]
BTC (4 mM)	25 ± 5 [b]
L-Alanine (20 mM)	92 ± 6

[a] The reaction mixture (0.2 mL) contained 10 mM L-leucine, 5 mM α-ketoglutarate, 100 mM potassium phosphate buffer (pH 7.4), 50 mM ammonium sulfate, 5 mM DTT, 5 μM PLP, 0.05 mM NADH, 0.95 U leucine dehydrogenase, and 90 ng (6.3 mU) of $BCAT_m$; 37°. Rates of formation of KIC were determined from 15 to 60 sec after the addition of $BCAT_m$. N = 3–5 separate determinations. All rates are calculated relative to that of the control reaction mixture (no addition). The data are expressed as means ± SEM.

[b] Different from the no addition with $P = 0.025$.

Table 5
Pyruvate formed from β-lyase substrates after incubation with $BCAT_c$ and low concentration of KIC [a]

L-Amino acid	Concentration (mM)	Pyruvate formed (nmol)			
		Time (min)			
		2	5	10	60
β-Chloro-L-alanine [b]	25	5.0	5.0	5.9	5.9
BTC	5	<0.5	<0.5	<0.5	<0.5
TFEC [b]	10	5.1	4.4	4.9	4.8
DCVC	5	3.7	4.5	4.9	4.9
CTFC	10	3.6	3.9	4.5	4.6

[a] $BCAT_c$ (5.35 μg, 460 mU) was incubated at 37° in 20-μL reaction mixtures containing 5 mM DTT, 5 μM PLP, 100 mM potassium phosphate buffer (pH 7.4), and the amino acids indicated. Due to carryover from the $BCAT_m$ storage buffer, the concentration of KIC in the reaction mixture was 0.1 mM. The blank contained reaction mixture plus boiled enzyme. At the times indicated, pyruvate in the 20-μL reaction mixture was determined as its 2,4-dinitrophenylhydrazone. Except in the case of BTC, where N = 4, data are the averages of duplicate determinations. The difference between the two values was <15% for all duplicate measurements.

[b] In a separate experiment, 9.0 and 8.5 nmol of pyruvate were formed from 25 mM β-chloro-L-alanine and TFEC, respectively (N = 2), in the presence of 0.87 U $BCAT_c$.

(Table 5). On average, the enzyme turned over only ~40–50 times per monomer before inactivation occurred with β-chloro-L-alanine. Because $BCAT_c$ was inhibited ≥95% after incubation with TFEC, DCVC, or CTFC, the data in Table 5 suggest on average about 40–45 turnovers per monomer inactivated with these cysteine *S*-conjugates as well.

The carryover of KIC in the enzyme storage buffer resulted in a concentration of KIC in the incubation mixture of 0.1 mM. Therefore, the possibility existed that some transamination might have occurred between the cysteine *S*-conjugates (or β-chloro-L-alanine) and KIC. However, the fact that (a) more pyruvate (5–6 nmol) was generated from the β-lyase substrates than the amount of KIC present in solution (2 nmol), and (b) ≥95% inactivation of enzyme occurred within 2 min showed that the β-elimination reaction with millimolar quantities of β-chloro-L-alanine, TFEC, DCVC, and CTFC was greatly favored over transamination. Transamination would have resulted in conversion of the enzyme to the PMP form, which would have been resistant to inactivation. In a separate experiment, $BCAT_c$-catalyzed L-leucine formation with the β-lyase substrates (β-chloro-L-alanine, TFEC, DCVC, or CTFC) and 10 mM KIC could not be detected (data not shown).

Due to the low number of turnovers per inactivation event and limitations in the sensitivity of the present assay procedures, measurement of the kinetic constants for the β-lyase substrates was not feasible for $BCAT_c$. On the other hand, it was possible to show that at 23° both β-chloro-L-alanine and TFEC are moderately good inhibitors of the $BCAT_c$-catalyzed L-leucine–α-ketoglutarate transamination reaction (Table 6). Interestingly, although BTC is not an irreversible inhibitor or β-lyase substrate, this cysteine *S*-conjugate binds effectively to $BCAT_c$. Thus, BTC strongly inhibited the $BCAT_c$-catalyzed transamination of leucine with α-ketoglutarate (Table 6). L-Alanine was not an effective inhibitor under the same conditions (Table 6).

Table 6
$BCAT_c$-catalyzed transamination of L-leucine with α-ketoglutarate in the presence of β-lyase substrates or L-alanine[a]

Addition	% Relative activity
None	[100 ± 4]
β-Chloro-L-alanine (10 mM)	53 ± 6[b]
TFEC (4 mM)	87 ± 3[b]
BTC (4 mM)	55 ± 3[b]
L-Alanine (20 mM)	95 ± 4

[a] The reaction mixture (0.2 mL) contained 10 mM L-leucine, 5 mM α-ketoglutarate, 100 mM potassium phosphate buffer (pH 7.4), 50 mM ammonium sulfate, 5 mM DTT, 5 μM PLP, 0.05 mM NADH, 0.95 U leucine dehydrogenase, and 0.16 μg (17.6 mU) of $BCAT_c$; 23°. Rates of formation of KIC were determined from 15 to 60 sec after the addition of $BCAT_c$. N = 3–5 separate determinations. All rates are calculated relative to the control (no addition). The data are expressed as means ± SEM.

[b] Different from no addition with $P = 0.025$.

4. Discussion

The BCAT isozymes by virtue of their ancestral lineage may be structurally poised to catalyze a very effective β-lyase reaction if confronted with an amino acid that contains a good leaving group in the β position such as β-chloro-L-alanine or a cysteine *S*-conjugate. There is a drawback, however, because the BCAT isozymes, and especially the cytosolic isozyme, are strongly susceptible to inactivation during turnover.

4.1. Interaction of BCAT isozymes with β-chloro-L-alanine

The results presented here show that β-chloro-L-alanine is a much more effective β-lyase substrate of human $BCAT_m$ than of pig heart cytAspAT and mitAspAT. The ratio of ~280 turnover to inactivation events per monomer for $BCAT_m$ in the presence of β-chloro-L-alanine is similar to that reported for inactivation of pig heart AlaAT [28] and AspAT isozymes [27] (~250–500 turnover events per monomer inactivated). However, the maximal rate of $BCAT_m$-catalyzed β-elimination with β-chloro-L-alanine of ~24% of the maximal rate of transamination with natural substrates (Table 3) is higher than the values observed with pig heart mitAspAT, cytAspAT, and AlaAT of ~2, ~9, and ~0.5%, respectively [27,28]. The K_m values reported for the binding of β-chloro-L-alanine to pig heart mitAspAT, cytAspAT, and AlaAT [27,28] are 50, 200, and ~0.1 mM, respectively, whereas the corresponding value for $BCAT_m$ was 0.6 mM (Table 3). The maximal specific activities for the "natural" transamination reactions catalyzed by pig heart AspAT, cytAspAT, and AlaAT are 200–500 U/mg versus about 100 U/mg for BCAT. Thus, the relative V_{max}/K_m value for $BCAT_m$-catalyzed β-elimination from β-chloro-L-alanine is >100 that exhibited by the pig heart AspAT isozymes, but roughly comparable to that of pig heart AlaAT.

The results with human $BCAT_c$ and β-chloro-L-alanine show that there are subtle differences between the human BCAT isozymes. Human $BCAT_c$ is more sensitive to inactivation by β-chloro-L-alanine than is human $BCAT_m$ (~45 vs ~280 turnover to inactivation events per monomer, respectively).

It was possible to obtain kinetic data at (or near) initial rate conditions for β-chloro-L-alanine as a β-lyase substrate of $BCAT_m$ when the reaction was carried out at 23°. Inactivation of $BCAT_c$ was, however, too rapid for such a calculation even at 23°. The kinetic data for $BCAT_m$ compare favorably with those obtained for L-leucine as an aminotransferase substrate. The V_{max} and apparent K_m values for the $BCAT_m$-catalyzed formation of pyruvate from β-chloro-L-alanine are 0.6 mM and 5.1 nmol/min/μg (Table 3), and are of the same order of magnitude as estimated for leucine transamination (i.e. 1.2 mM [65] and 21 nmol/min/mg of protein [Table 3]). Thus, the

V_{max}/K_m value for β-chloro-L-alanine as a β-lyase substrate is comparable to that exhibited by L-leucine as an aminotransferase substrate. Moreover, at a concentration (10 mM) comparable to that of L-leucine in the aminotransferase assay, β-chloro-L-alanine markedly inhibits (~85%) the initial rates of $BCAT_m$-catalyzed transamination between L-leucine and α-ketoglutarate (Table 4). L-Alanine is not a substrate of rat or human $BCAT_m$ [65], and this amino acid is a poor inhibitor of the $BCAT_m$-catalyzed transamination of L-leucine (Table 4). On the other hand, L-amino acids with longer aliphatic side chains (L-norvaline, L-norleucine) are comparable to glutamate in activity [65]. The present data suggest that β-chloro-L-alanine can fit into the active site of $BCAT_m$ as a very good mimic of L-norvaline.

4.2. Interaction of BCAT isozymes with cysteine S-conjugates

Both human BCAT isozymes catalyze a β-lyase reaction with cysteine *S*-conjugates, and competing transamination reactions cannot be detected. The V_{max} value for TFEC as a β-lyase substrate exhibited by $BCAT_m$ (0.3 nmol/min/μg, at 23°) is considerably less (1–2%) than the rate of optimal L-leucine transamination catalyzed by this enzyme (21 nmol/min/mg at 23°) (Table 3). However, the affinity of $BCAT_m$ for TFEC at the active site (apparent K_m value of 8.4 mM) is not greatly different from that of L-leucine (apparent K_m ~1.0 mM, [12]). V_{max} and K_m data were not obtained for BTC as a β-lyase substrate. However, the data in Table 4 show that BTC is an effective inhibitor of $BCAT_m$. Under the conditions noted in Table 4, BTC appears to bind more tightly than leucine to the active site of $BCAT_m$.

Studies on the inhibition of the $BCAT_c$-catalyzed L-leucine aminotransferase reaction suggest that β-chloro-L-alanine and BTC bind to the active site of the enzyme about as effectively as L-leucine, and that TFEC binds only slightly less effectively (Table 6). Comparison of Tables 4 and 6 shows that BTC is a somewhat better inhibitor of the $BCAT_m$-catalyzed transamination reaction than of the $BCAT_c$-catalyzed transamination reaction.

Rat liver mitAspAT catalyzes a β-lyase reaction with halogenated cysteine *S*-conjugates [34]. As with the BCAT isozymes, syncatalytic inactivation of mitAspAT by the cysteine *S*-conjugates occurs. There are, however, differences between the BCAT isozymes and mitAspAT. Some transamination competes with the β-lyase reaction at the active site of mitAspAT, and the inactivation of mitAspAT is more pronounced if the α-keto acid substrate, α-ketoglutarate, is also present [34]. Transamination does not compete with the cysteine *S*-conjugate β-lyase reaction catalyzed by the BCAT isozymes. Moreover, $BCAT_m$ is partially protected against inactivation at high concentrations of the α-keto acid substrate KIC (compare Tables 1 and 2). The apparent protection by KIC may be due to the formation of an abortive (unproductive) complex between the PLP form of the enzyme and α-keto acid substrate, which hinders binding of the β-lyase substrate. Such abortive complexes are known to occur for other aminotransferases [69].

4.3. Mechanism of inactivation by β-lyase substrates

The human BCAT isozymes, like most cysteine *S*-conjugate β-lyases characterized thus far, including kynureninase [70], cytAspAT [33], mitAspAT [34], and AlaAT [33], but not cytosolic glutamine transaminase K (cytGTK) [71], are inactivated syncatalytically by the β-lyase substrates. It was suggested originally that inactivation of cytAspAT by β-chloro-L-alanine is due to alkylation of a crucial residue at the active site by aminoacrylate [26,27], and the same mechanism may hold for inactivation of bacterial D-amino acid aminotransferase by β-chloro-D-alanine [24]. Alternatively, aminoacrylate generated from the β-lyase reaction may interact with the co-factor forming a PLP–pyruvate aldol adduct that remains attached at the active site as observed for inactivation of both glutamate decarboxylase and pig heart cytAspAT by the β-lyase substrate, L-serine *O*-sulfate [72,73]. A third possible route for inactivation by the halogenated cysteine *S*-conjugates (but not by β-chloro-L-alanine or BTC) involves thioacylation by the eliminated sulfur-containing fragment. In this regard, it should be noted that kidney mitAspAT is thioacylated after rats are administered TFEC [53]. A thioacylation reaction might also occur with the BCAT isozymes. Whether PLP or an active site residue such as Lys^{202} or Cys^{315}/Cys^{318} in the BCAT isozymes is modified remains to be determined.

4.4. Comparison of the BCAT isozymes

The specificity and kinetic constants of the two isozymes for the natural substrates are generally similar [65,66]. Moreover, the present studies show that both enzymes (a) possess cysteine *S*-conjugate β-lyase activity, (b) are inactivated during turnover of cysteine *S*-conjugates, and (c) are unable to catalyze transamination when confronted with halogenated cysteine *S*-conjugates such as DCVC or TFEC (present work). Nevertheless, the present finding that BTC is a β-lyase substrate of $BCAT_m$, but not of $BCAT_c$, despite the fact that this cysteine *S*-conjugate can bind to both active sites, indicates that there are subtle differences in active site topology between the two enzymes. This conclusion is in agreement with a previous study showing that the neuroactive drug gabapentin [1-(aminomethyl)cyclohexane acetic acid] inhibits $BCAT_c$, but not $BCAT_m$ [19]. The findings suggest that the topology of the hydrophobic pocket that binds the hydrophobic side groups of the natural substrates differs between $BCAT_c$ and $BCAT_m$.

4.5. Do BCATs contribute to the toxicity of halogenated cysteine S-conjugates?

It is reasonable to assume that, like the human BCAT isozymes, the rat BCAT isozymes can also catalyze

cysteine *S*-conjugate β-lyase reactions with halogenated cysteine *S*-conjugates. The present results show that human $BCAT_m$ has a β-lyase activity (with 20 mM TFEC; 37°) of ~6.1 nmol/min/U. Rat kidney contains ~3.5 U $BCAT_m$/g wet weight. [74]. Therefore, if the activities toward TFEC are similar for the rat and human enzymes, the TFEC lyase activity of rat kidney mitochondria due to $BCAT_m$ would be ~21.4 nmol/min/g wet weight. The cysteine *S*-conjugate β-lyase activity in rat kidney mitochondria (20 mM TFEC; 37°) is ~0.58 μmol/min/g wet weight [34]. Therefore, the contribution of $BCAT_m$ to the TFEC lyase activity in rat kidney mitochondria may be about 4% of the total. A cysteine *S*-conjugate β-lyase (BTC as substrate) has been purified from human kidney [75]. The same study showed BTC lyase activity to be present in human kidney mitochondria [75]. The previous finding of relatively high $BCAT_m$ activity in human kidney [74], coupled with the present finding that $BCAT_m$ catalyzes a BTC–lyase reaction (Table 1), show that a portion of the BTC lyase activity previously detected in human kidney must have been due to $BCAT_m$.

With the notable exception of liver, most rat tissues contain $BCAT_m$, but the distribution of $BCAT_c$ is much more restricted [12]. In the rat, the enzyme was originally thought to occur only in brain, placenta, and ovary [12]. However, our recent study of the cellular localization of $BCAT_c$ in rat tissues using isoenzyme-specific antibodies indicates that $BCAT_c$ is also present in peripheral nerves [76]. In the central nervous system, immunocytochemical studies of primary cultures of rat brain cells show that $BCAT_c$ is the only BCAT isozyme in neurons, whereas $BCAT_m$ is localized primarily in astroglia [18,21]. In rat brain cortex and cerebellum, $BCAT_c$ is present only in neurons [22]. Dichloroacetylene (formed by the alkaline breakdown of trichloroethylene) is readily converted to DCVC and is neurotoxic to experimental animals [77,78]. Interestingly, a post-mortem study of an individual acutely poisoned by trichloroethylene/dichloroacetylene revealed neuronal degeneration within the brain stem sensory nucleus of the trigeminal nerve and degeneration of axons within its tract [79]. DCVC is readily transported into the brain on the leucine (L) carrier [80]. This raises the possibility that toxic cysteine *S*-conjugates formed directly in the brain or imported into the brain can inactivate the BCAT isozymes in the brain or in peripheral nerves, and this may contribute to the neurotoxicity.

In 1986, Stevens *et al*. [71] identified cytGTK (a freely reversible glutamine (methionine) phenylalanine aminotransferase (e.g. Ref. [81]) as a major cysteine *S*-conjugate β-lyase of rat kidney. Due to competing transamination reactions, the cysteine *S*-conjugate β-lyase activity of the rat kidney cytGTK isolated by Stevens *et al*. [71] has a relatively strong requirement for added α-keto acid (e.g. α-keto-γ-methiolbutyrate, the α-keto acid analogue of methionine). This is not the case with human $BCAT_m$ and $BCAT_c$. Thus, the ratio of β-elimination/transamination with halogenated cysteine *S*-conjugates is much more favorable for the BCAT isozymes than for rat kidney cytGTK.

In a recent survey, we noted that at least nine PLP-containing enzymes (including $BCAT_m$ and $BCAT_c$) possess cysteine *S*-conjugate β-lyase activity [63]. Certainly, under optimal assay conditions cytGTK exhibits the highest specific activity as a cysteine *S*-conjugate β-lyase of all the purified enzymes surveyed [63]. Most of the GTK activity in rat kidney [81] is associated with the cytosolic fraction (as assessed by the standard phenylalanine–α-keto-γ-methiolbutyrate transaminase assay). The GTK activity in rat kidney mitochondria (10% of the total in kidney; [81]) does not co-purify with cysteine *S*-conjugate β-lyase activity [82]. Moreover, administration of TFEC to rats results in labeling of several mitochondrial (but not cytosolic) proteins [53–55]. Therefore, it seems likely that cytGTK is not the cysteine *S*-conjugate β-lyase responsible for targeting of renal mitochondria. But why then does cytGTK in the presence of halogenated cysteine *S*-conjugates not cause toxicity by generating a fragment that can thioacylate cytosolic proteins? The concentrations of phenylpyruvate and α-keto-γ-methiolbutyrate (α-keto acid substrates of cytGTK) are low in rat kidney and liver[3] (<1 μM). Therefore, the lack of action of cytGTK as a cysteine *S*-conjugate β-lyase *in vivo* may be due to a strong tendency to catalyze transamination of the cysteine *S*-conjugate and to low levels of α-keto acid substrate, thereby "tying up" the enzyme in the PMP form. In agreement with this idea, α-keto-γ-methiolbutyrate exacerbates the toxicity of DCVC to isolated rat kidney proximal tubules and mitochondria, but only at extremely high (non-physiological) levels [83].

Cysteine *S*-conjugate β-lyase activity in rat kidney mitochondria has been detected in both the outer membrane [83,84] and matrix [85]. Under the conditions of our assay (TFEC as substrate), most of the cysteine *S*-conjugate β-lyase activity (83%) of rat kidney is in the cytosolic fraction [34]. Of the cysteine *S*-conjugate β-lyases in the mitochondrial fraction (17%), about 15–20% could be accounted for by mitAspAT [34]. Thus, the contribution of mitAspAT to the total (i.e. cytosolic plus mitochondrial) rat kidney cysteine *S*-conjugate β-lyase activity (when assayed under optimal conditions) is rather small (about 3%). However, the toxicological importance of mitAspAT may be much more important than previously recognized. Of interest is the finding that aconitase [55] and the E2 and E3 subunits of α-ketoglutarate dehydrogenase (but not those of pyruvate dehydrogenase complex) [54] in rat kidney mitochondria are labeled and inactivated by TFEC. We have suggested that a supramolecular complex formed between mitAspAT and various components of the tricarboxylic acid (TCA) cycle

[3] A.J.L. Cooper, unpublished observation.

("metabolon") facilitates *toxicant channeling* from mitAspAT to enzymes of the TCA cycle [54,55,63].

As with mitAspAT, the importance of $BCAT_m$ as a bioactivation enzyme may be greater than appreciated from its relative activity as a cysteine *S*-conjugate β-lyase. The E3 subunits of the rat kidney branched-chain α-keto acid dehydrogenase complex are thioacylated by a TFEC fragment [86]. We have found that $BCAT_m$ forms a complex *in vitro* with the branched-chain α-keto acid dehydrogenase complex.[4] Therefore, one can hypothesize that the specific labeling of the E3 subunit of the branched-chain α-keto acid dehydrogenase complex by a TFEC-derived fragment may be due to toxicant channeling involving a $BCAT_m$ metabolon.

4.6. Conclusion

$BCAT_m$ and $BCAT_c$ should now be added to the list of PLP-containing enzymes that can catalyze cysteine *S*-conjugate β-lyase reactions. No single cysteine *S*-conjugate β-lyase is responsible for all of the toxicity of halogenated cysteine *S*-conjugates. Most likely the cysteine *S*-conjugate-induced toxicity in a given organ is a reflection in part of (a) the ability of that organ to accumulate the cysteine *S*-conjugate pro-toxicant in its mitochondria, (b) the complement of PLP-containing enzymes (particularly aminotransferases) within the mitochondria that can catalyze a cysteine *S*-conjugate β-lyase reaction, (c) effectiveness of natural substrates to protect by competing with cysteine *S*-conjugates at the active site, (d) the ability of the mitochondrial PLP-containing enzymes (such as $BCAT_m$ and mitAspAT) to provide a reactive fragment (toxicant) to nearby susceptible proteins, and possibly (e) the ability of the toxic cysteine *S*-conjugate to induce self-destruction of a susceptible PLP-containing enzyme. In regard to the last point, the BCAT isozymes, particularly $BCAT_c$, are very susceptible to inactivation by toxic halogenated cysteine *S*-conjugates. Finally, the present results showing that BTC binds to both $BCAT_m$ and $BCAT_c$, but undergoes a β-lyase reaction only at the active site of $BCAT_m$ may provide a basis for further studies on the active site topologies of the BCAT isozymes.

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