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Use of sulfhydryl reagents to investigate branched chain α-keto acid transport in mitochondria

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Abstract

The goal of this paper was to determine the contribution of the mitochondrial branched chain aminotransferase (BCATm) to branched chain α-keto acid transport within rat heart mitochondria. Isolated heart mitochondria were treated with sulfhydryl reagents of varying permeability, and the data suggest that essential cysteine residues in BCATm are accessible from the cytosolic face of the inner membrane. Treatment with 15 nmol/mg *N*-ethylmaleimide (NEM) inhibited initial rates of α-ketoisocaproate (KIC) uptake in reconstituted mitochondrial detergent extracts by 70% and in the intact organelle by 50%. KIC protected against inhibition suggesting that NEM labeled a cysteine residue that is inaccessible when substrate is bound to the enzyme. Additionally, the apparent mitochondrial equilibrium KIC concentration was decreased 50–60% after NEM labeling, and this difference could not be attributed to effects of NEM on matrix pH or KIC oxidation. In fact, NEM was a better inhibitor of KIC oxidation than rotenone. Measuring matrix aspartate and glutamate levels revealed that the effects of NEM on the steady-state KIC concentration resulted from inhibition of BCATm catalyzed transamination of KIC with matrix glutamate to form leucine. Furthermore, circular dichroism spectra of recombinant human BCATm with liposomes showed that the commercial lipids used in the reconstituted transport assay contain BCAT amino acid substrates. Thus BCATm is distinct from the branched chain α-keto acid carrier but may interact with the inner mitochondrial membrane, and it is necessary to inhibit or remove transaminase activity in both intact and reconstituted systems prior to quantifying transport of α-keto acids which are transaminase substrates.

Keywords: Mitochondria; Transport; Transamination; Branched chain α-keto acid

1. Introduction

The first step in the catabolism of the essential amino acids, leucine, valine, and isoleucine, is transamination to their respective branched chain α-keto acids. The transport of these metabolites across the inner mitochondrial membrane is catalyzed by the branched chain α-keto acid transporter which has been characterized kinetically in rat heart mitochon-

Abbreviations: BCATm, mitochondrial branched chain aminotransferase; BCAT, branched chain aminotransferase; KIC, α-ketoisocaproate; NEM, *N*-ethylmaleimide; mersalyl, *O*-(3-hydroxymercuri-2-methoxypropyl)carbamylphenoxyacetate; pCMB, *p*-chloromercuribenzoate; pCMBS, *p*-chloromercuribenzene sulfonate; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 3-(*N*-morpholino)-propanesulfonate; AOA, amino-oxyacetate

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dria [1,2]. The substrate specificity of the exchange reaction and pH dependence suggest that the branched chain α-keto acid carrier is distinct from the pyruvate transporter; nevertheless, both carriers appear to catalyze uptake of their respective monocarboxylic acids via a proton symport mechanism [1,3]. Subsequently, the branched chain α-keto acid transporter was solubilized in functionally active form from rat heart mitochondria and shown to be sensitive to sulfhydryl reagents [4]. Evidence was presented [5] suggesting that BCATm could be the branched chain α-keto acid transporter characterized previously [1]; however, it remains to be established whether BCATm actually functions as a transporter in intact heart mitochondria.

Organic mercurials and maleimides have been the most common reagents employed to characterize mitochondrial anion carriers both in situ [6–14] and using recombinant transport proteins in reconstitution systems [15–22]. The effects of these reagents on transporter function have suggested that cysteine residues are important for these proteins, hypotheses which have been addressed through site-directed mutagenesis [19,23–26]. With the recent availability of the complete yeast genome sequence, genetic and biochemical approaches continue to be used to identify new members of the mitochondrial carrier superfamily [27–31]. Although recent evidence suggests the existence of a mitochondrial translocase for acylcarnitine intermediates derived from branched chain amino acid oxidation [32], the transport proteins for branched chain α-keto acids and pyruvate have not been reported for either yeast or mammalian systems. Therefore, we utilized the availability of recombinant BCATm [33] and its sensitivity to sulfhydryl reagents [5,34] to assess the possible role of this protein in mitochondrial branched chain α-keto acid transport.

2. Materials and methods

2.1. Preparation of mitochondria

Rat heart [35] and liver [36] mitochondria were prepared from male Sprague–Dawley rats (250–300 g) using mannitol sucrose isolation medium (0.225 M mannitol, 0.075 M sucrose, 5 mM MOPS, 0.1 mM EDTA, pH 7.0). Respiratory control ratios were measured using glutamate and malate as substrates as described by Hutson et al. [37], and no preparations with a ratio of <6 were used. Protein was determined by the biuret reaction in the presence of 0.25% sodium deoxycholate using crystalline bovine albumin as a standard.

2.2. Incubation of mitochondria with sulfhydryl reagents

All incubations and washes were performed with mannitol sucrose isolation medium. Freshly prepared heart or liver mitochondria (20 mg/ml) were incubated with sulfhydryl reagents at 4°C for 15 min. After incubation with NEM, the reaction was terminated by the addition of a 10-fold excess of DTT, and diluted 40-fold with isolation medium containing 3 mM DTT. The mitochondrial solution was centrifuged at $8000\times g$ for 10 min, washed twice, and resuspended at a concentration of 20 mg protein/ml. After incubation with pCMB, pCMBS, or mersalyl, mitochondria were diluted 40-fold with isolation medium and washed twice. In some experiments, α-ketoisocaproate (KIC, 2 mM) was added 5 min before NEM. Control incubations were conducted under identical conditions in the absence of sulfhydryl reagents.

2.3. Measurement of mitochondrial metabolites

Rat heart mitochondria were extracted either with 2% sulfosalicylic acid and assayed for glutathione using the recycling assay essentially as described by Tietze [38] or with 2% perchloric acid and assayed for aspartate, glutamate, and α-ketoglutarate using standard fluorimetric methods [39,40]. NADH fluorescence (excitation: 340 nm, emission: 465 nm) was monitored using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Instruments, Rochester, NY).

2.4. Measurement of α-keto acid transport in intact mitochondria and determination of intramitochondrial pH

Uptake of α-keto acids into heart or liver mitochondria was measured at 8°C as described previ-

ously [1,2]. Briefly, an aliquot of mitochondria was added to a chamber containing 140 mM KCl, 20 mM MOPS (pH 7, 8°C), 10 mM succinate, 12 μM rotenone, 7% dextran, and [1-^{14}C]sucrose or $^{3}H_2O$. At selected times, samples were taken, and the mitochondria were separated from the media by rapid centrifugation through silicone oil (83.5 parts Dow Corning 550 and 16.5 parts Dow Corning 200). Rates of transport were calculated by determining the initial rate of α-keto acid uptake. Alternatively, transport rates were calculated assuming a first order approach to a final equilibrium value for the internal α-keto acid concentration [2,41]. With this method [41], $\ln([A_t]/([A_t]-[A_i]))$ is plotted versus time where $[A_t]$ is the internal concentration of α-keto acid at steady state and $[A_i]$ is the internal concentration at time t. The first order rate constant for efflux, k, is the slope of this line. Since at steady state α-keto acid efflux is equal to uptake, the rate of α-keto acid transport can be calculated using the following equation: $v = k[A_t]$. It is assumed that the rate of uptake is constant over time and efflux is first order.

Matrix pH was determined using the weak acid 5,5′-dimethyl[2-^{14}C]oxazolidine-2,4-dione as described [2], and matrix water was determined using $^{3}H_2O$ and [1-^{14}C]sucrose. Measurements of mitochondrial pH were made in samples of mitochondria incubated under the same experimental conditions as in the transport or labeling experiments.

2.5. Solubilization of the mitochondria and reconstitution of α-keto acid transport activity

Mitochondria (approximately 40–45 mg protein) were extracted with 1% Triton X-114 as described previously [4] without addition of KIC or cardiolipin. Phospholipid vesicles were prepared as described [4,42] except vesicles were formed by sonication using a Branson Sonifier 250 (Branson Ultrasonics, Danbury CT) at an output setting of 1.6–2 and a duty cycle of 70–90%. Depending on the volume of vesicles required, either a 0.5-inch diameter disruptor horn with flat tip or a double stepped microtip was used. Resuspended lipids were placed on ice and sonicated 3–5 min then allowed to cool for 1–2 min. These steps were repeated until the lipid suspension cleared (~10–20 min). Then proteoliposomes were formed by adding aliquots of the Triton X-114 mitochondrial extract to the phospholipid vesicles followed by freeze–thaw sonication [4,42]. In a previous description of this procedure [4], detergent extraction was carried out in the presence of KIC. Subsequently, the proteoliposomes were passed over Dowex-1(X8) anion exchange resin to remove excess KIC prior to use in the transport assay. For the experiments presented in this paper, KIC was not included in the extraction, thus the proteoliposomes were not subjected to Dowex resin treatment prior to measuring KIC transport. pCMB sensitive [1-^{14}C]KIC and [1-^{14}C]pyruvate transport activities were measured as described [4,5]. The sulfhydryl reagent pCMB was also used as an inhibitor stop for pyruvate, because previous studies demonstrated that it was more effective than the noncompetitive pyruvate transport inhibitor α-cyano-3-hydroxycinnamic acid [43]. Aliquots of each reaction mixture were placed on separate Dowex-1(X8) columns; the liposomes were eluted, and radioactivity was quantified as described [4,42]. In the detergent extracts, protein was measured using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA).

2.6. Measurement of α-keto acid oxidative decarboxylation under transport assay conditions

Oxidative decarboxylation of [1-^{14}C]KIC or [1-^{14}C]-pyruvate was measured in the transport buffer at 8°C as described by Hutson et al. [37] except $^{14}CO_2$ was trapped with 0.25 ml tissue solubilizer (DuPont/NEN, Wilmington, DE). Data were corrected for the efficiency of the CO_2 trap with an average recovery of 85%.

2.7. Determination of branched-chain aminotransferase activity

Mitochondrial branched-chain aminotransferase activity was measured at 37°C as described previously [43]. The final assay mixture contained 50 mM potassium phosphate buffer, pH 7.8 (KOH), 50 μM pyridoxal phosphate, 0.4% CHAPS, 2 mM DTT, 1 mM α-[1-^{14}C]ketoisovalerate, and 12 mM isoleucine. CHAPS was not added in assays of Triton X-114 mitochondrial extracts, and DTT was omitted from the samples and control assays for all sulfhydryl reagents except NEM.

2.8. Lipid extraction and thin-layer chromatography

3-*sn*-Phosphatidylcholine from either dried egg yolk or soybean (Fluka, Ronkonkoma, NY) was suspended in H_2O at 50 mg lipid/ml, sonicated to form liposomes, and extracted using the method of Bligh and Dyer [44]. After centrifugation, the aqueous phase was removed, dried under vacuum, and resuspended in 10% methanol. Samples, amino acids, and phospholipid standards were applied to Silica Gel G plates (20×20 cm, Analtech, Newark, DE) and developed in either chloroform/methanol/ammonium hydroxide (2:2:1, v/v) or methylethylketone/pyridine/H_2O/glacial acetic acid (70:15:15:2, v/v) [45]. Plates were air dried at room temperature $\geq$1 h, and amino acids were visualized with ninhydrin (88% acetone, 2% acetic acid, 0.2% ninhydrin, 0.1% cadmium acetate). Plates were also sprayed with a 55% sulfuric acid solution containing 0.6% (w/v) potassium dichromate, and phospholipids were visualized by charring.

2.9. Circular dichroism spectroscopy

Near UV circular dichroism spectroscopy was performed as described previously [33] with recombinant human BCATm in the absence and presence of phosphatidylcholine liposomes. The enzyme was converted to the pyridoxal phosphate form by incubation with 1 mM KIC for 1 h or to the pyridoxamine phosphate form by incubation with 5 mM leucine for 1 h followed by dialysis to remove substrate [33]. Liposomes were formed by sonication as described above, and the final solution (1 ml) contained 10 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM DTT, 5 mg/ml lipid, and 0.3 mg recombinant human BCATm.

2.10. Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test. Significance was reached at $P < 0.005$.

2.11. Chemicals and reagents

L-[1-^{14}C]Leucine was obtained from either Amersham Pharmacia Biotech (Piscataway, NJ) or DuPont/NEN (Wilmington, DE). L-[1-^{14}C]Valine was obtained from American Radiolabeled Chemicals (St. Louis, MO). Radiolabeled KIC and α-ketoisovalerate were synthesized from leucine and valine, respectively, essentially as described by Rüdiger [46]. 5,5′-Dimethyl[2-^{14}C]oxazolidine-2,4-dione was obtained from Amersham Pharmacia Biotech. NEM, pCMB, pCMBS, and mersalyl were obtained from Sigma (St. Louis, MO). AOA was obtained from Aldrich (Milwaukee, WI). Pure 3-*sn*-phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL).

3. Results and discussion

3.1. Effect of sulfhydryl reagents on transamination and KIC transport

To better define the localization of BCATm in mitochondria and to determine its actual contribution to mitochondrial branched chain α-keto acid transport, intact rat heart mitochondria were incubated with permeable and impermeable sulfhydryl reagents. Then the mitochondria were extracted with detergent, BCAT activity was measured in the extracts and KIC transport activity was measured after reconstitution in proteoliposomes (Table 1). NEM was the most effective reagent with 80% inhibition of aminotransferase and transport activities observed at a concentration of 15 nmol/mg mitochondrial protein (Table 1). Similar concentrations of mersalyl and pCMB also inhibited both activities by 60–70% whereas a higher concentration of pCMBS (100 nmol/mg mitochondrial protein) was required to achieve the same degree of inhibition. With the exception of the polar pCMBS, both enzyme activities were inhibited in parallel, and pCMBS appeared to have a significantly greater effect on transport activity than on transaminase activity ($P < 0.005$).

As also shown in Table 1, measurements of total mitochondrial glutathione confirmed that only NEM was freely permeable to the inner mitochondrial membrane. Reports with liver [47] and heart [48] mitochondria have shown previously that the mercurial reagents, pCMB, pCMBS, and mersalyl, are generally considered impermeable. Addition of DTT to the reaction mixtures did not affect changes in glu-

Table 1
Effect of sulfhydryl reagents on rat heart mitochondrial BCATm activity, reconstituted KIC transport activity, and total glutathione content

-−SH Reagent	Mitochondrial detergent extracts		
	BCAT activity (% control)	KIC transport (% control)	Total glutathione (% control)
NEM	19 ± 1	20 ± 3	7 ± 1
Mersalyl	28 ± 1[a]	24 ± 0.4[a]	101 ± 5
pCMB	39 ± 1[b]	30 ± 2[b]	92 ± 9
pCMBS	36 ± 4[c]	22 ± 3[c]	88 ± 3

Preparation of mitochondria, incubation with sulfhydryl reagents, extraction procedures, and measurement of BCAT, reconstituted KIC transport activity and glutathione content are described in Section 2. For NEM, the average and range of two mitochondrial preparations, one with triplicate and one with quadruplicate samples, are shown. Values for mersalyl are means ± S.D. from quadruplicate samples in one mitochondrial preparation. For pCMB and pCMBS, means ± S.E. are presented from three mitochondrial preparations. Control transaminase and KIC transport activities were 127 ± 24 ($n = 9$) and 3 ± 0.3 ($n = 9$) nmol/min per mg mitochondrial protein, respectively. Values for glutathione are the mean ± S.E. from 3–5 mitochondrial preparations. Control preparations contained 5.9 ± 0.3 ($n = 8$) nmol glutathione/mg mitochondrial protein.
[a]25 nmol/mg mitochondrial protein.
[b]10 nmol/mg mitochondrial protein.
[c]100 nmol/mg mitochondrial protein

tathione levels but reversed the inhibition by pCMB, pCMBS, and mersalyl restoring BCAT activity to ≥90% of control values (data not shown). Therefore, the results suggested that the reactive SH group(s) in BCATm was accessible from the cytosolic side of the inner mitochondrial membrane, and that both transport and transaminase activities were affected by these reagents.

Next, NEM (15 nmol/mg) was used to determine the effect of inhibiting BCATm on the kinetics of KIC uptake in isolated rat heart mitochondria, and the results were compared with liver mitochondria which do not contain BCATm [49]. At this concentration of NEM, only the phosphate carrier should be affected [8] whereas the pyruvate, dicarboxylate, tricarboxylate, and α-ketoglutarate carriers would not be inhibited [47,50]. Pyruvate uptake, which should not be affected by this concentration of NEM [50], was also measured in selected experiments. Table 2 shows the effect of NEM on initial rates of KIC uptake in intact mitochondria and after detergent extraction of the mitochondria and recon-

Table 2
Summary of NEM inhibition of the initial rates of α-keto acid transport in heart and liver mitochondria and a reconstituted system

Mitochondria	Monocarboxylate transport			
	Isolated mitochondria (% control)		Reconstitution system (% control)	
	Pyruvate	KIC	Pyruvate	KIC
Heart				
Control	100	100	100	100
NEM	93 ± 3	48 ± 3	108 ± 9	29 ± 4
KIC+NEM	ND[a]	75 ± 4	ND	73 ± 10
Liver				
Control	100	100	–	–
NEM	101 ± 1	125 ± 8	–	–

Freshly isolated mitochondria were incubated with NEM (15 nmol/mg) and either used to determine α-keto acid uptake or extracted with detergent as described in Section 2. KIC and pyruvate concentrations ranged from 20 to 100 μM. The results are the mean ± S.E. for 3–6 mitochondrial preparations.
[a]ND, not determined.

stitution of transport activity in proteoliposomes. Initial rates of pyruvate uptake in liver and heart mitochondria were unaffected by NEM treatment, whereas initial rates of KIC uptake in liver mitochondria were increased after NEM treatment (Table 2). We have also observed that addition of NEM (20–50 nmol/mg protein) directly to the transport assay stimulated the rate of α-ketoisovalerate uptake in liver mitochondria preparations by 16–57% (data not shown). In heart mitochondria, initial rates of KIC uptake were inhibited 50% by NEM treatment, and addition of KIC before NEM provided substantial protection against inhibition (Table 2). These results were consistent with the idea that BCATm plays a role in KIC transport in situ.

As suggested by the time course of KIC uptake with and without NEM (Fig. 1), NEM appeared to affect not only the initial rate of uptake but also the steady-state level of KIC ($[KIC_t]$). The data were fit to the equation $\ln([A_t]/([A_t]-[A_i])) = kt$, and the rate of α-keto acid uptake was calculated using the equation $v = k[A_t]$ [41]. Inhibition of KIC uptake by NEM would be expected to lower the amount of active transporter resulting in a lowered rate of KIC uptake, a longer time to reach equilibrium, and a lower V_{max}, but would not be expected to affect $[KIC_t]$, which is dependent on the ΔpH across the inner mitochondrial membrane. Nonetheless, calculated and observed $[KIC_t]$ values were decreased approximately 50% compared with untreated controls (Fig. 1). Table 3 shows the ratios of the mean equilibrium KIC and pyruvate concentrations in NEM-treated heart and liver mitochondria compared with control mitochondria. The ratios of mean steady-state pyruvate concentrations were similar in both types of mitochondria, whereas the $[KIC_t]$ ratio was significantly higher than 1.0 in liver and lower than 1.0 in heart ($P < 0.005$). Because the steady-state distribution of the KIC anion should be proportional to the pH gradient, the ΔpH was measured in the control and NEM-treated mitochondria (Table 3). Although heart mitochondria respiring on succinate maintain a significantly higher ΔpH than liver mitochondria respiring on succinate [2], the experimentally determined matrix pH during the transport experiments was comparable in control and NEM-treated mitochondria (Table 3). Moreover, the similarity in the ratio of steady-state pyruvate concentrations in control and NEM-treated heart and liver mitochondria is consistent with the lack of change in ΔpH.

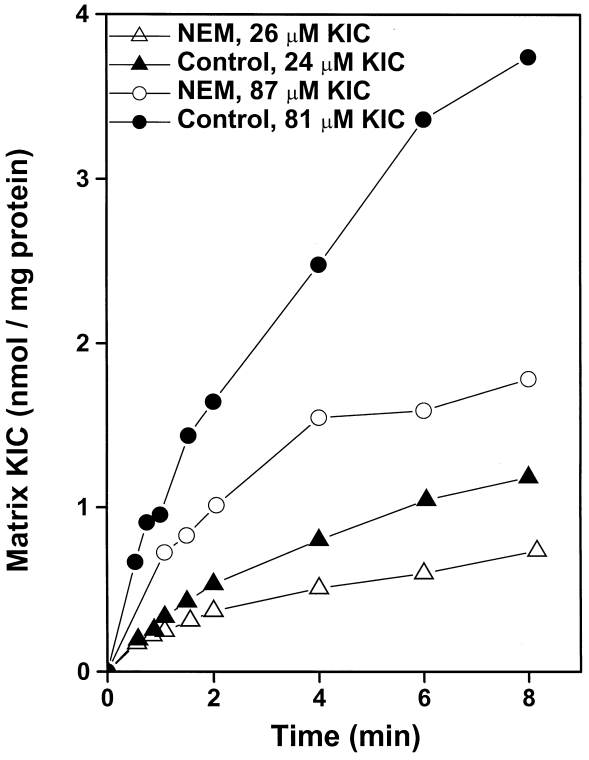

Fig. 1. Effect of NEM treatment on the time course of α-ketoisocaproate (KIC) uptake in isolated heart mitochondria. Freshly isolated mitochondria were treated with NEM (15 nmol/mg protein), and mitochondrial uptake of [1-^{14}C]KIC was measured at 8°C as described in Section 2. The medium pH was 7.1. Calculated k_{efflux} and steady-state matrix KIC concentrations ($[KIC_t]$) were: control (▲), 0.18 min^{-1} and 1.52 nmol/mg protein; NEM treated (△), 0.29 min^{-1} and 0.71 nmol/mg protein; control (●), 0.22 min^{-1} and 4.48 nmol/mg protein; NEM treated (○), 0.38 min^{-1} and 1.64 nmol/mg protein. KIC concentrations are shown in the figure.

Differences in the uptake curves could also result from metabolism of the α-keto acids. Because a specific inhibitor of the branched chain α-keto acid dehydrogenase enzyme complex is not available, metabolism of the α-keto acids was blocked by conducting the experiments at 8°C in the presence of rotenone which blocks oxidation of NADH, a product of branched chain α-keto acid oxidative decarboxylation. Direct measurement of [1-^{14}C]KIC (30 μM) oxidation in the transport buffer was 0.75 ± 0.21 nmol/mg protein per 10 min in heart ($n = 5$) and 0.83 ± 0.15 nmol/mg protein/10 min in liver ($n = 4$), which indicated that rotenone alone is

Table 3
Influence on NEM on calculated equilibrium matrix α-keto acid concentration and matrix pH in intact mitochondria

	$[KIC_t]$ ratio in NEM-labeled vs. control mitochondria	Matrix pH	
		Control	NEM
Heart mitochondria		8.78 ± 0.06	8.84 ± 0.09
KIC	0.50 ± 0.05[a]		
Pyruvate	1.17 ± 0.11		
Liver mitochondria		8.47 ± 0.03	8.50 ± 0.03
KIC	1.94 ± 0.26[a]		
Pyruvate	1.14 ± 0.07		

Heart and liver mitochondria were prepared and treated with NEM, KIC uptake was measured, and equilibrium matrix concentrations of KIC ($[KIC_t]$) and pyruvate were calculated as described in Section 2. Matrix pH was measured using 5,5′-dimethyl oxazolidine-2,4-dione [2], and medium pH was 7.08. Matrix H_2O was the same in control and NEM samples, and the mean value was 0.93 ± 0.03 μl/mg mitochondrial protein. KIC was 30 μM, and pyruvate was 100 μM. Means ± S.E. from 4–8 experiments are shown.
[a] $P < 0.005$.

not sufficient to block KIC metabolism. However, KIC oxidation was depressed in NEM-treated heart and liver mitochondria to 0.11 ± 0.04 nmol/mg protein per 10 min ($n = 5$) and 0.12 ± 0.04 nmol/mg protein per 10 min ($n = 4$), respectively. Sensitivity to sulfhydryl reagents has been reported for the branched chain α-keto acid dehydrogenase enzyme complex [51]; therefore, NEM inhibition of KIC oxidation in liver would be consistent with the higher $[KIC_t]$ values (Table 3) and the increased initial rates of KIC uptake (Table 2) observed after NEM treatment of liver mitochondria. However, this would not explain the lower equilibrium KIC concentration found in heart mitochondria. Since BCATm is not present in liver but is found in heart, the higher $[KIC_t]$ seen in control heart mitochondria could result from conversion of KIC to leucine which is subsequently trapped in the matrix in the absence of an exchangeable amino acid. Bachowska-Mac et al. [52] have shown that transamination was the driving force for KIC uptake across the plasma membrane of neuroblastoma cells, and it has been suggested that neutral amino acid transport in liver mitochondria occurs through an exchange mechanism [53,54]. To test this possibility, heart mitochondrial aspartate and glutamate concentrations were measured under the conditions used in the uptake assay. KIC transamination with glutamate would be expected to stimulate conversion of glutamate to α-ketoglutarate in the mitochondrial matrix. Addition of KIC to control mitochondria lowered matrix aspartate levels 30–40% whereas glutamate levels were unaffected, suggesting that KIC transamination with glutamate shifts the aspartate aminotransferase reaction toward the utilization of aspartate and the production of glutamate. Addition of KIC to NEM-treated mitochondria did not affect matrix glutamate or aspartate levels; therefore, it appears that BCATm-catalyzed transamination of KIC to leucine is occurring in control mitochondria and that the radioactive leucine remains in the matrix. Thus, the assumption that $v = k[A_t]$ is incorrect, because $[KIC_t]$ is overestimated. These results explain the apparent decrease in the equilibrium KIC concentration after NEM treatment and suggest that KIC transport measured after the inhibition of BCATm by NEM more accurately reflects the actual branched chain α-keto acid carrier activity.

3.2. Analysis of lipids used for reconstitution of KIC transport

The time course results obtained with isolated mitochondria may be explained if BCATm was catalyzing transamination of matrix KIC with endogenous mitochondrial substrates; however, reconstituted KIC transport activity was also inhibited by NEM (Tables 1 and 2). Therefore, we investigated the possibility that transamination was occurring in the proteoliposomes. Intact mitochondria as well as reconstituted mitochondrial detergent extracts contain numerous proteins other than BCATm that may have an effect on measured branched chain α-keto acid transport; therefore, to simplify the system, pu-

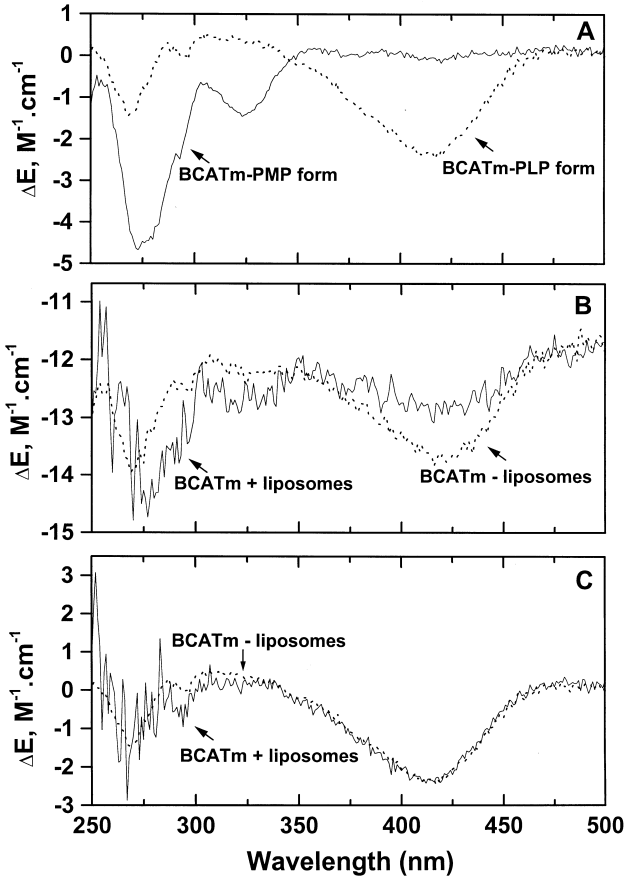

Fig. 2. Near UV circular dichroism spectroscopy of human BCATm in phosphatidylcholine liposomes. Samples were prepared as described in Section 2. The pyridoxal and pyridoxamine phosphate forms of human BCATm were prepared by incubating the enzyme with the appropriate substrate followed by dialysis. Near UV circular dichroism spectra were measured in a 1 cm quartz cuvette and are the average of four accumulations. (A) Spectra were taken in the absence of liposomes. The dotted line represents the pyridoxal phosphate form, and the solid line represents the pyridoxamine phosphate form of BCATm. (B,C) The dotted line represents the pyridoxal phosphate form in the absence of liposomes, and the solid line represents the BCATm spectrum in the presence of egg yolk (B) or soybean (C) liposomes.

rified recombinant human BCATm was used. This enzyme has been cloned, overexpressed, and characterized [33]. An amino acid sequence comparison [55] shows that rat and human BCATm are 82% identical, and like the rat enzyme [34], human BCATm is inhibited 79 ± 2% ($n = 4$) by 1 mM NEM. Additionally, human BCATm appears to catalyze KIC transport when added to egg yolk liposomes. The apparent rate of KIC uptake (100 μM KIC) was 4 ± 0.5 μmol/min per mg protein ($n = 12$).

BCATm is a ping-pong enzyme and contains pyridoxal-5′-phosphate as a cofactor which alternates between two forms, the pyridoxal and the pyridoxamine form [56]. Addition of an amino acid substrate results in the formation of an external aldimine-amino acid-pyridoxal phosphate complex which is then converted to the pyridoxamine form of the cofactor and α-keto acid product. This process is reversible in the presence of a second α-keto acid substrate which converts the cofactor back to its pyridoxal form producing the corresponding amino acid. Circular dichroism spectroscopy was used to determine whether or not there were changes in protein conformation or form of the enzyme cofactor in recombinant human BCATm when added to liposomes. Human BCATm was converted to the pyridoxal form of the cofactor, and the spectrum is shown by the dotted line in Fig. 2A. This form is characterized by a strong peak of ellipticity at 421 nm and weaker ellipticity at 277 nm [33]. The enzyme was also converted to the pyridoxamine form as shown by the solid line in Fig. 2A. This form exhibits weak ellipticity at 326 nm and a strong peak of ellipticity at 277 nm [33]. Addition of the pyridoxal form of BCATm to egg yolk liposomes resulted in conversion of approximately 60% of the enzyme to the pyridoxamine form (Fig. 2B) as suggested by the disappearance of ellipticity at 421 nm, the appearance of a weaker peak at 326 nm, and an increase in ellipticity around 277 nm [33]. However, no spectral changes were seen with vesicles from either purified phosphatidylcholine (data not shown) or crude phosphatidylcholine from soybean (Fig. 2C). These results suggested that a substrate amino acid was present in the egg yolk lipids.

To characterize possible endogenous amino acids in the commercial lipid, the egg yolk and soybean lipids were extracted with methanol and chloroform [44]. The aqueous phase of the lipid extracts, which contained water soluble compounds, was subjected to thin-layer chromatography. Numerous amine containing compounds from both the egg yolk and soybean lipids co-migrated with the amino acid standards (Fig. 3A); however, fewer compounds were seen in the soybean sample. Egg yolk phosphatidylcholine appeared to contain isoleucine and leucine (Fig. 3A); however, the hydrophobic amino acid

tryptophan migrated along with the branched chain amino acids when the solvent system was chloroform/methanol/ammonium hydroxide. Using a more hydrophobic solvent system, spots correlating to both leucine and isoleucine were seen in the egg yolk lipids but not in the soybean lipids (Fig. 3B). A spot that appeared to co-migrate with the glutamate standard was found in the egg yolk lipids developed in the hydrophobic solvent system (Fig. 3B) but not in the more hydrophilic system (Fig. 3A). Quantifying radiolabeled glutamate added to the lipids prior to extraction revealed that $<3\%$ partitioned in the lipid phase. Because the lipids are prepared commercially by organic extraction, it is unlikely that glutamate was present; however, these results indicate that hydrophobic amino acids, such as the branched chain amino acids or alanine, may be present in the phospholipids used routinely for mitochondrial transporter reconstitution (for review see [57]).

This conclusion is supported by activity measurements using human BCATm reconstituted in egg yolk, soybean, and purified phosphatidylcholine lipids. KIC transport and transamination were compared in the absence and presence of either NEM or the general transaminase inhibitor, amino-oxyacetate (AOA). Because the specific activity of the enzyme is dependent on both the amount of KIC added and the concentration of amino acid in the liposomes, both activities were measured under identical conditions, and NEM or AOA (1 mM) was added 5 min before addition of radiolabeled KIC. Both KIC transport and transamination were similar in egg yolk lipids, 3.00 ± 0.01 ($n=2$) and 4.0 ± 0.1 ($n=2$) μmol/min per mg, respectively, and both activities were inhibited 88–89% by NEM. AOA inhibition could not be measured in the transaminase assay, because the reagent is known to form an adduct with KIC in the presence of acid which cannot be decarboxylated [43]; however, KIC transport in the egg yolk lipids was decreased $>99\%$ after incubation with AOA. Finally, in soybean and purified phosphatidylcholine lipids, both transport and transamination were $<1\%$ of the activity seen with egg yolk lipids. These results indicate that transamination can occur in the reconstituted KIC transport assay and further support the conclusion that amino

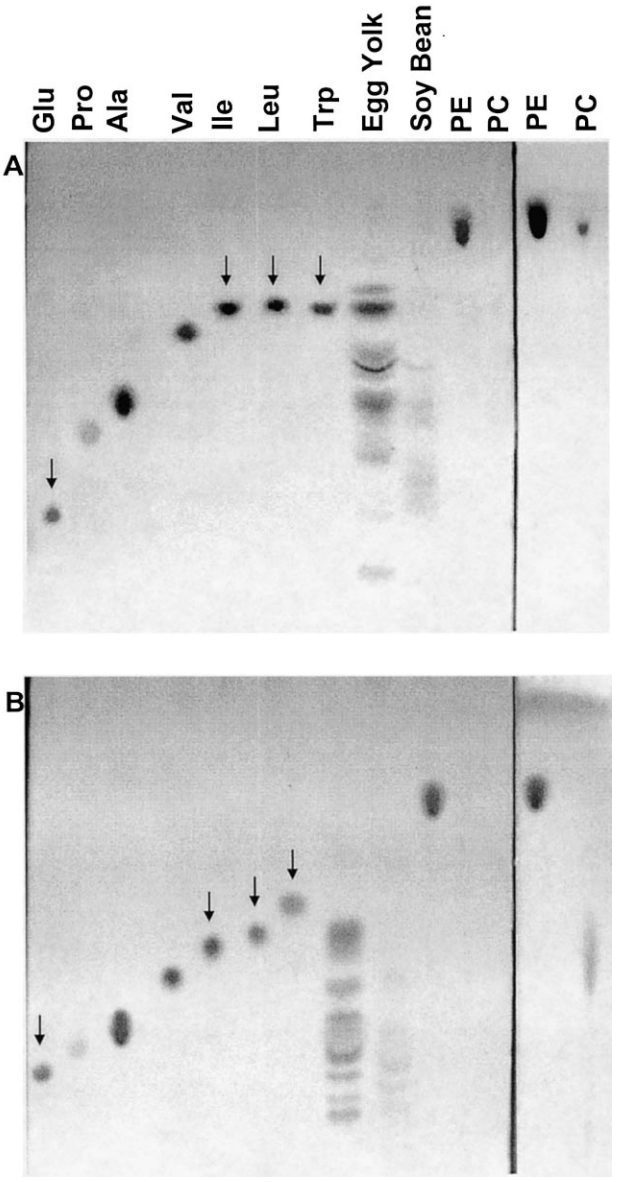

Fig. 3. Thin-layer chromatography of the aqueous layer from egg yolk and soybean liposome extracts. Egg yolk and soybean liposomes were prepared and extracted as described in Section 2. Amino acid standards are listed along with the phospholipid standards phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Various amino acids are highlighted with arrows. On the left side, amino acids were visualized with ninhydrin spray, and on the right side, phospholipids were sprayed with a sulfuric acid solution and charred. (A) Plate was developed in chloroform/methanol/ammonium hydroxide (2:2:1, v/v) for 45 min. (B) Plate was developed in methylethylketone/pyridine/H_2O/acetic acid (70:15:15:2, v/v) for 65 min.

acid substrates are present in commercial preparations of the egg yolk lipids.

4. Conclusions

The goal of this paper was to determine the contribution of BCATm to mitochondrial branched chain α-keto acid transport. Analysis of KIC uptake in isolated mitochondria showed that BCATm utilization of endogenous mitochondrial substrates altered apparent equilibrium concentrations of KIC, and the enzyme's reaction with endogenous substrates in commercial lipid preparations could be monitored by CD spectroscopy. Moreover, apparent reconstituted KIC transport was decreased $>99\%$ by the transaminase inhibitor, AOA. These results indicate that BCATm-catalyzed transamination can affect transport measurements and that BCATm and the branched chain α-keto acid carrier are separate proteins. Therefore, transaminase activity must be inhibited when measuring transport of potential α-keto acid substrates in either intact organelles, such as mitochondria, yeast, or cell lines, or in reconstitution systems. In lieu of an enzyme specific inhibitor, AOA may be a useful reagent in these types of experiments. Addition of this compound to heart mitochondria reduced BCATm-catalyzed transamination to below detectable levels without affecting branched chain α-keto acid oxidation [43]. Thus, this inhibitor appears not to affect transport and may be effective in studies of other α-keto acid carriers such as the pyruvate transporter in intact systems.

The presence of amino acid substrates in commercial lipid preparations also provides an explanation for previous results obtained with mitochondrial extracts. The described procedure [4] for detergent extraction of the branched chain α-keto acid transporter from mitochondria used 5 mM KIC in the extraction buffer and soybean lipids for reconstitution of KIC transport activity in proteoliposomes. Subsequently, the proteoliposomes were passed over Dowex anion exchange resin to remove excess KIC prior to use in the transport assay [4]. This step would also tend to remove BCATm from the proteoliposomes, since the enzyme is an anion at neutral pH. This procedure was later modified such that the Dowex resin step was omitted, and activity was reconstituted in egg yolk lipids resulting in a $\geq$10-fold increase in apparent KIC transport [5]. This result may now be explained by the presence of BCATm and amino acid substrates in the egg yolk lipid samples. Additionally, transport and transaminase activities in the proteoliposomes were reduced 80–90% after one passage over the anion exchange resin, and the resulting KIC transport activity (0.5 nmol/min per mg mitochondrial protein) was comparable to that observed in either NEM or AOA-treated mitochondria (0.6–0.7 nmol KIC/min per mg protein). Thus, various chromatographic methods such as ion exchange over Dowex or Amberlite resin also may be effective tools in removing unwanted enzyme activities from reconstitution mixtures.

Finally, the inhibition of BCATm within the intact mitochondria after treatment with impermeable sulfhydryl reagents is an intriguing result. Analysis of the primary sequence of rat or human BCATm with the PSORT II program [58,59] to detect transmembrane segments predicts only one potential membrane spanning region within the BCATm monomer. Therefore, one may speculate that BCATm loosely associates with the inner mitochondrial membrane or is bound to a second membrane anchored protein which permits labeling of reactive cysteine residues within BCATm from the cytosolic face of the inner mitochondrial membrane. This phenomenon has been reported for the mitochondrial aspartate aminotransferase which has been shown to bind to inverted mitochondrial inner membrane vesicles [60,61] possibly via a second membrane anchoring protein [62]; however, further studies have not been reported. Thus, additional experimental approaches will be required to determine the precise mitochondrial localization of BCATm.

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