Abstract: Astroglia-rich primary cultures and brain slices rapidly metabolize branched-chain amino acids (BCAAs), in particular leucine, as energy substrates. To allocate the capacity to degrade leucine oxidatively in neural cells, we have purified β-methylcrotonyl-CoA carboxylase (β-MCC) from rat liver as one of the enzymes unique for the irreversible catabolic pathway of leucine. Polyclonal antibodies raised against β-MCC specifically cross-reacted with both enzyme subunits in liver and brain homogenates. Immunocytochemical examination of astroglia-rich rat primary cultures demonstrated the presence of β-MCC in astroglial cells, where the enzyme was found to be located in the mitochondria, the same organelle that the mitochondrial isoform of the BCAA aminotransferase (BCAT) is located in. This colocalization of the two enzymes supports the hypothesis that mitochondrial CAT is the isoenzyme that in brain energy metabolism prepares the carbon skeleton of leucine for irreversible degradation in astrocytes. Analysis of neuron-rich primary cultures revealed also that the majority of neurons contained β-MCC. The presence of β-MCC in most neurons demonstrates their ability to degrade the α-ketoisocaproate that could be provided by neighboring astrocytes or could be generated locally from leucine by the action of the cytosolic isoform of CAT that is known to occur in neurons. Key Words: Astroglia—Energy metabolism—Enzyme purification—Immunocytochemistry—Leucine. J. Neurochem. 74, 1059–1067 (2000).

Plasma branched-chain amino acids (BCAAs), in particular leucine, are readily transported across the blood–brain barrier (Oldendorf, 1971; Smith et al., 1987). In brain, BCAAs are believed to subsist at least two functions: (a) BCAAs are actively degraded in brain slices and astroglia-rich primary cultures (Chaplin et al., 1976; Bixel and Hamprecht, 1995) as valuable metabolic “fuel” molecules in brain energy metabolism. (b) BCAAs are believed to be important for nitrogen turnover by contributing their amino group to the glial glutamate pool (Yudkoff et al., 1983, 1994). The first reaction in metabolism of BCAAs—reversible transamination of BCAAs to their cognate α-keto acids (Roswell and Turner, 1963; Ichihara and Koyama, 1966; Taylor and Jenkins, 1966; Aki et al., 1967)—is catalyzed in brain by the BCAA aminotransferase (BCAT), either the cytosolic (BCATc) or the mitochondrial (BCATm) isoenzyme (Hall et al., 1993). Most tissues metabolizing BCAAs express exclusively BCATm (Hutson, 1988). BCATc, which is found only in a few organs, is the predominant isoenzyme in brain (Hall et al., 1993). Recently, the cellular location of BCATm and BCATc in cultured neural cells has been studied immunocytochemically to contribute to the clarification of the metabolic function(s) of the two BCAT isoenzymes in neural tissues. BCATm was predominantly found in astroglial cells, whereas BCATc was present in neurons, oligodendroglial cells, and also, to a variable extent, astroglial cells (Bixel et al., 1996, 1997). Based on this isoenzyme pattern, the authors suggested that BCATm is part of the mitochondrial catabolic pathway in astroglial cells that degrades BCAAs for generation of brain energy. Also, in supplementing the glutamate/glutamine cycle, BCAAs may be involved in the α-ketoisocaproate/leucine cycle, which is thought to transfer nitrogen from glutamatergic neurons to astrocytes (Yudkoff et al., 1996b). Yudkoff et al. (1983, 1994) and Hutson et al. (1998) hypothesized that BCAAs also function as an amino group donor for glutamate synthesis from α-ketoglutarate in astrocytes, which subsequently could promote the glutamine transfer from these cells to neurons.
The hypothesis that BCATm initiates in mitochondria of astroglial cells the irreversible breakdown of BCAAs would be substantiated by the colocalization of BCATm with the enzymes of the further catabolic pathway. As a first step to allocate cellularly the catabolic pathway of leucine, we purified β-methylcrotonyl-CoA carboxylase (EC 6.4.1.4; β-MCC), as one of the enzymes unique for this pathway (Alberts and Vagelos, 1972). The branched-chain α-keto acid dehydrogenase (BCKDH), which catalyzes the rate-limiting step in leucine oxidation (Harper et al., 1984), was not chosen for this purpose because this enzyme complex also irreversibly decarboxylates the α-keto acids derived from the two other BCAAs, isoleucine and valine. β-MCC is a biotin-containing (Lynen et al., 1961) mitochondrial (Hector et al., 1980) enzyme that catalyzes ATP-dependent carboxylation of a β-methyl carbon of the branched acyl-CoA substrate, resulting in activation of the methyl branch, thus facilitating further degradation of the carbon skeleton. We report here the purification of β-MCC from rat liver, the generation of an antisera against β-MCC, and the use of these antibodies in the immunocytochemical analysis of astroglia-rich and of neuron-rich primary cultures from rat brain.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco (Eggenstein, Germany). All cell culture plasticware and 96-well Nunc-immuno plates were from Nunc (Wiesbaden, Germany). Acrylamide, Bio-Rad protein assay, and N,N,N’,N’-tetramethylethylenediamine were obtained from Bio-Rad Laboratories (München, Germany). ATP, avidin, 5-bromo-4-chloro-3-indolyl phosphate, creatine phosphate, dithiothreitol, 4-nitro blue tetrazolium chloride, 4-nitrophenyl phosphate, the molecular mass marker set (Combiotheck), sodium dodecyl sulfate (SDS), Tris, and Triton X-100 were from Boehringer (Mannheim, Germany). Creatine kinase (rabbit muscle), N,N,N’,N’-tetramethylthelylenediamine, and Tween 20 were from Serva (Heidelberg, Germany). Nitrocellulose filter sheets and ultra-free MC 10,000 NMWL microconcentrators were purchased from Millipore (Eschborn, Germany). Biotin, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, Freund’s incomplete adjuvant, β-methylcrotonyl-CoA, and poly-o-lysine hydrobromide were purchased from Sigma (Deisenhofen, Germany). NaH¹⁴CO₃, (1.92 GBq/mmol) was from Amersham Buchler (Braunschweig, Germany). Center wells were from Landgraf Laborgeräte (Langenhagen, Germany). Scintillation fluid was obtained from Roth (Karlsruhe, Germany). Freund’s complete adjuvant was from Calbiochem (Gießen, Germany). DE-52 cellulose was from Whatman (Maidstone, Kent, U.K.), and CNBr-activated Sepharose was from Pharmacia (Freiburg, Germany). The mouse anti-vimentin monoclonal antibody was a gift from Dr. Mary Osborn (Göttingen, Germany). Alkaline phosphatase-conjugated goat anti-rabbit IgG, alkaline phosphatase-conjugated streptavidin, fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG, and tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG were obtained from Sigma. All other chemicals, of highest purity available, were obtained from E. Merck (Darmstadt, Germany).

**β-MCC assay**

The catalytic activity of β-MCC was assayed by measuring the incorporation of H¹⁴CO₃⁻ into β-methylcrotonyl-CoA, generating ¹⁴C-labeled β-methyl-glutaryl-CoA in a modification of the method described by Lau et al. (1980). The assay mixture consisted of 240 μl of reaction solution [70 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl₂, 2 mM ATP, 0.53 mM β-methylcrotonyl-CoA, 0.05 mM NaH¹⁴CO₃ (3.1 KBq/μmol), 10 mM creatine phosphate, and 5 μM creatine kinase] and 10–20 μl of an enzyme solution. After 20 min the reaction was terminated by addition of 200 μl of 1 M HCl. After removing unreacted H¹⁴CO₃⁻ by shaking and absorption with filters soaked with 200 μl of 5 M NaOH, the radioactivity remaining in the sample was determined using a scintillation spectrometer (model 1214 Rackbeta; LKB, Gräfelfing, Germany). The assay conditions guarantee a proportionality between incorporation of H¹⁴CO₃⁻ into β-methylcrotonyl-CoA and the time of incubation as well as between the incorporation and the amount of enzyme or homogenate supernatant.

**Purification of β-MCC**

β-MCC was purified from frozen livers of rats, which were obtained from the animal facilities of our institute. Because of the instability of the enzyme, which was found to be stable only after an almost pure enzyme preparation was obtained, the protocol for the isolation of β-MCC was kept as short as possible. Therefore, the preparation of the mitochondrial fraction as a first step (Lau et al., 1979) was omitted, as already performed by others (Lau et al., 1980; Oei and Robinson, 1985). The original protocol of Oei and Robinson (1985) was modified considerably. After homogenization of 100 g of rat liver in 250 ml of buffer H (4°C; pH 8.0) containing 100 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% (vol/vol) glycerol in a Potter–Elvehjem-type glass/Teflon homogenizer, the suspension was centrifuged at 48,000 g for 20 min.

The resulting supernatant was subjected to ammonium sulfate fractionation in the range of 30–55% saturation. After centrifugation (10,000 g, 4°C, 10 min) the pellet was resuspended in a minimal volume of buffer A [20 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% (vol/vol) glycerol, pH 7.5] and dialyzed overnight against 10 L of buffer A at 4°C. The enzyme solution was applied to a chromatography column (4 × 20 cm) containing DE-52 cellulose equilibrated with buffer A. The column was washed with buffer A, and β-MCC activity was eluted using a linear gradient of 0.0–0.5 M KCl in buffer A. Fractions containing significant β-MCC activity were pooled. The enzyme was further purified by affinity column chromatography on avidin-Sepharose, which was prepared as described by Henrickson et al. (1979). The enzyme solution was loaded onto the avidin-Sepharose column (2 × 6 cm) equilibrated with buffer AK (buffer A containing 0.5 M KCl). After washing with buffer AK the column was eluted with 0.25 mg/ml biotin in buffer AK. The fractions containing β-MCC activity were dialyzed against buffer B and applied to a second anion exchange chromatography column (1 × 20 cm) containing DE-52 cellulose equilibrated with buffer A. The column was washed with buffer A, and the enzyme was eluted with a linear gradient of 0.0–0.3 M KCl in buffer A. The peak of enzyme activity (at 0.10–0.15 M KCl) was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), and fractions containing only the two subunits of β-MCC were pooled and concentrated in an ultracentrifugation cell using a Filtron microconcentrator.
Preparation of anti-β-MCC antisera

For immunization, four rabbits (Chincilla bastards) were initially injected subcutaneously with 200 μg of purified β-MCC in 500 μl of 20 mM Tris-HCl (pH 7.5) mixed with 50 μl of complete Freund’s adjuvant. Boosting was carried out at 4-week intervals using 100 μg of β-MCC in Freund’s incomplete adjuvant, and antibody titers were determined by ELISA. After the second booster two rabbits showed titers of >10,001. Western blot analysis demonstrated that one of the two antisera examined recognized exclusively the two subunits of β-MCC. The other antisera also stained a protein band of 130 kDa corresponding to pyruvate carboxylase. The sera of the two remaining rabbits were discarded because their titers were much lower.

ELISA

ELISAs were performed by coating 96-well plates overnight at 4°C with 100 ng of β-MCC per well diluted in sodium borate buffer (125 mM sodium borate and 75 mM NaCl, pH 8.4). Nonspecific protein binding sites on the plates were blocked with 1% (wt/vol) milk powder in borate buffer (2 h, room temperature) before incubation with β-MCC antisera (1:200, 2 h, room temperature). The wells were washed twice with 0.05% (wt/vol) Tween 20 in borate buffer, and then anti-rabbit IgG conjugated with alkaline phosphatase (1:3,000) was added (1 h, room temperature). The wells were washed twice with 0.05% (wt/vol) Tween 20 in borate buffer and then incubated for 30 min with alkaline phosphatase substrate solution (1 mg/ml 4-nitrophenyl phosphate in 100 mM glycine/NaOH, 1 mM MgCl₂, and 1 mM ZnCl₂, pH 10.4). The absorbance at 405 nm was determined in a Titertek Multiscan MCC/340 ELISA reader.

Immunoblotting

Western blots (Burnette, 1981) were performed on proteins separated by SDS-PAGE (Laemmli, 1970). Transfer onto nitrocellulose was performed at a current of 0.3 A for 12 h in transfer buffer (25 mM Tris and 192 mM glycine, adjusted to pH 9.0 with KOH). Nitrocellulose strips were treated with 1% (wt/vol) milk powder in incubation buffer [20 mM Tris-HCl, 150 mM NaCl, and 0.02% (wt/vol) Tween 20, pH 7.4] at room temperature for 2 h to block unspecified protein binding sites. After washing the strips three times with incubation buffer, they were treated with the β-MCC antisera (diluted 1:200 in incubation buffer) at room temperature for 2 h. Unbound antibodies were removed by washing three times with incubation buffer, and the strips were incubated with secondary alkaline phosphatase-conjugated anti-rabbit IgG diluted 1:3,000 in incubation buffer. After washing the strips with incubation buffer, color development was carried out in staining buffer [100 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, 365 μM 5-bromo-4-chloro-3-indolyl phosphate, and 45 μM 4-nitroblue tetrazolium chloride, pH 8.9].

Primary cultures

Astroglia-rich primary cultures derived from the brains of newborn Wistar rats (bred in the animal facilities of our institute) were prepared and cultured as described previously (Hamprecht and Löfler, 1985). Cells were seeded into plastic culture dishes 50 mm in diameter at a plating density of 2.5–3.0 × 10⁶ viable cells per dish. The cells were incubated at 37°C in a humidified atmosphere of 10% CO₂/90% air in a nutrient medium containing 90% DMEM/10% FCS, 20 units/ml penicillin, and 20 μg/ml streptomycin sulfate. Every seventh day the medium was renewed using 90% DMEM/10% FCS. The vast majority of the cells in the cultures were astroglial cells (Reinhart et al., 1990).

Brains from Wistar rats of embryonic age 16 days were removed and dissected as described previously by Löfler et al. (1986). Cells were plated in plastic dishes (50 mm in diameter, 3 × 10⁶ cells per dish) precoated with 5 mg/ml poly-γ-lysine hydrobromide in 0.15 M borate buffer (pH 8.4) and cultured as described (Löfler et al., 1986).

Immunocytochemistry

Cells grown to confluency on glass coverslips (22 × 22 mm) during 5–8 days after seeding were prepared for immunocytochemistry essentially as reported previously (Hamprecht and Löfler, 1985). For staining, cells were fixed at room temperature using 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 10 min. Fixed cells were washed twice in PBS for 5 min and once in PBS containing 0.1% (wt/vol) glycine for 5 min. Cells were permeabilized in PBS containing 0.3% (wt/vol) Triton X-100 for 10 min. Immunofluorescent double-labeling was carried out using the indirect two-antibody method with FITC-labeled anti-mouse IgG for glial fibrillary acidic protein (GFAP) or vimentin and TRITC-labeled anti-rabbit IgG for β-MCC as secondary antibodies. Antibodies were diluted in PBS containing 0.3% (wt/vol) Triton X-100 and 10% (vol/vol) normal goat serum. The coverslips carrying the cells were then placed in a humidified chamber and treated with a mixture of the two primary antibodies for 2 h and with the two secondary antibodies for 1 h. After each incubation, the cells were washed twice in PBS containing 0.1% (wt/vol) Triton X-100. Coverslips were mounted, cells down, using 50% (vol/vol) glycerol in PBS. The preparations were viewed by glycerol immersion optics using a Zeiss fluorescence microscope (IM35) with a Plan-Neofluar 25× objective.

The specificity of the β-MCC staining of the cultures was tested by immunoabsorption experiments. The suitable antigen concentration for incubation of the antisera was determined using ELISAs. Each well of the 96-well plates was coated overnight with 100 ng of purified β-MCC. To evaluate the amount of β-MCC needed for completely blocking the antigen binding sites of the β-MCC antibodies, the antisera against β-MCC (diluted 1:200; final volume 80 μl) was incubated overnight at 4°C with 5 ng–10 μg of β-MCC. Blocking of unspecific binding sites, incubation of first and secondary antibodies, and color development were performed as described above. Preincubation of the β-MCC antisera (diluted 1:200) with 8 μg of β-MCC decreased the intensity of color development to <5%. Therefore, before being used as negative controls for immunocytochemistry, the β-MCC antisera (diluted 1:200) was preincubated with 8 μg of purified β-MCC at 4°C for 12 h.

Protein determinations

Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Purification of β-MCC and preparation of specific antibodies against β-MCC were undertaken with the aim of performing immunocytochemical studies to localize this enzyme in cultured astroglial cells and neurons. Among the enzymes unique for the leucine catabolic pathway we have chosen to purify β-MCC, a biotin-containing enzyme, because it was expected to be highly
TABLE 1. Purification of rat liver β-MCC

| Purification step                              | Volume (ml) | Protein (mg) | Total units (U) | Specific activity (mU/mg) | Yield (%) | Purification factor |
|-----------------------------------------------|-------------|--------------|----------------|--------------------------|-----------|-------------------|
| 48,000-g supernatant                          | 345         | 10,109       | 12.2           | 1.12                     | 100       | 1.0               |
| (NH₄)₂SO₄ fractionation (30–55%)               | 78          | 3,736        | 5,213          | 1.40                     | 43        | 1.2               |
| Anion exchange chromatography (DE-52 cellulose) | 205         | 825          | 2,500          | 1.95                     | 21        | 2.5               |
| Affinity chromatography (avidin-Sepharose)    | 60          | 4.4          | 1,189          | 270                      | 9.7       | 223               |
| Anion exchange chromatography (DE-52 cellulose) | 5           | 0.4          | 200            | 500                      | 1.6       | 413               |

FIG. 1. Purification of β-MCC from rat liver. Samples of each purification step were electrophoretically separated using an 8% SDS-polyacrylamide gel. Lanes 1–7 show Coomassie Blue staining (Laemmli, 1970) of the molecular mass marker proteins (lanes 1 and 7; molecular mass in kDa on the ordinate: phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa). The isolated protein was judged to be highly purified based on SDS-PAGE (Fig. 1). Coomassie Blue staining of the gel revealed two protein bands with molecular masses of ~72 and 60 kDa (Fig. 1, lane 6), corresponding to the two nonidentical subunits of β-MCC (Lau et al., 1980; Oei and Robinson, 1985). The 72-kDa protein was identified as the biotin-containing subunit by western blot analysis using streptavidin-alkaline phosphatase for detection (Fig. 1, lane 8).

Antisera against β-MCC were raised by immunizing four rabbits with the purified enzyme and testing their antibody titers using ELISAs. One rabbit developed an antiserum strongly cross-reacting in western blot analysis with rat β-MCC in liver and brain homogenates (Fig. 2, lanes 4 and 5). The antiserum specifically labeled the α and β subunit of the enzyme, as indicated by two protein bands with molecular masses of ~60 and 72 kDa visualized by the alkaline phosphatase reaction.

The capability of astroglial cells to degrade leucine oxidatively was examined immunocytochemically using astroglia-rich primary cultures (Fig. 3). In double-staining experiments, the cells were exposed to β-MCC antiserum in combination with antibodies against the glial marker GFAP (Bignami et al., 1972; Bock et al., 1977; present study, Fig. 3A–C). Staining of the cultures displayed an intensive β-MCC immunoreactivity of flat and irregularly shaped cells, which were identified as astroglial cells by colocalization with GFAP. The β-MCC fluorescence signal was located in the cytoplasm, whereas the areas of the nuclei were unstained. The punctate and rod-like appearance of the β-MCC staining

FIG. 2. Determination of the specificity of the β-MCC antiserum. Rat liver and rat brain homogenates were electrophoretically separated on an 8% SDS-polyacrylamide gel. Lanes 1–3 show Coomassie Blue staining (Laemmli, 1970) of the molecular mass marker proteins (lane 1; molecular mass in kDa on the ordinate: phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa), of proteins of a rat liver homogenate (40 µg; lane 2), and of the proteins of a rat brain homogenate (40 µg; lane 3). After electroblotting onto nitrocellulose, lane 4 (rat liver homogenate; 6 µg of protein) and lane 5 (rat brain homogenate; 9 µg of protein) were sequentially incubated with antiserum against β-MCC (diluted 1:200), antirabbit IgG conjugated with alkaline phosphatase, and a solution containing nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.
confirms the mitochondrial localization of this enzyme (McFarlane and von Holt, 1969; Hector et al., 1980; present study, Fig. 3A). The majority of β-MCC-positive cells were GFAP-expressing astroglial cells. However, several GFAP-negative cells were also present in the cultures, which displayed intensive β-MCC immunoreactivity. These cells had small and round somata with thin and extended processes. As indicated by preliminary double-labeling experiments using β-MCC antiserum together with anti-galactocerebroside (Ranscht et al., 1982) and A2B5 (Eisenbarth et al., 1982) antibodies (photomicrographs not shown), some of the GFAP-negative cells expressing β-MCC represent oligodendroglial and O2A progenitor cells (Raff et al., 1983), respectively.

To investigate the presence of β-MCC in neurons, neuron-rich primary cultures were stained with β-MCC antiserum (Fig. 4). Most neurons showed strong immunoreactivity for β-MCC, with the fluorescence signal being more intensive in regions around the nuclei (Fig. 4A and C). Some neurites labeled strongly for β-MCC, whereas others showed only weak staining, probably owing to mitochondria being located to a variable extent in neuronal processes. Apparently, not all neurons in these cultures showed the same staining intensity. Instead, a neuronal subpopulation exhibited only low immunoreactivity for β-MCC (Fig. 4C). Staining the neuron-rich primary cultures investigated in this study with an antibody against the neuronal marker GAP-43 (Kierstein et al., 1996) indicated that the majority of the cells were GAP-43-positive and therefore represent neurons (photomicrographs not shown). The staining procedure necessary for GAP-43 labeling requires another method for fixation of the cells, which does not allow binding of anti-β-MCC antibodies to their antigen (data not shown).
Therefore, the simultaneous application of anti-$\beta$-MCC and anti-GAP-43 antibodies in a double-staining experiment could not be performed. GAP-43-negative cells in these cultures were to a large extent astroglial cells. Consequently, the antiserum against $\beta$-MCC was applied in combination with an anti-vimentin antibody to label “contaminating” astroglial cells in all developmental stages (Dahl et al., 1981; present study, Fig. 4B and D). Mitochondria of the vimentin-positive astroglial cells were labeled for $\beta$-MCC in the distinctive pattern as already described above.

In control experiments in which the $\beta$-MCC antiserum was replaced by preimmune serum, only background staining was observed (photomicrographs not shown). The specificity of the $\beta$-MCC antiserum was demonstrated by preabsorbing the antiserum with purified $\beta$-MCC before using it for immunocytochemistry. Figure 3D shows immunocytochemical staining of an astroglia-rich primary culture exposed to $\beta$-MCC antiserum that was preabsorbed with purified $\beta$-MCC. The staining for $\beta$-MCC of the cells was completely prevented; only background staining was observed. When the antigen $\beta$-MCC was omitted during the preabsorption step and astroglia-rich primary cultures were treated with $\beta$-MCC antiserum, the characteristic punctate staining pattern of $\beta$-MCC, as seen in Fig. 3A, was preserved (photomicrographs not shown).

**DISCUSSION**

In the present study we immunocytochemically localized in astroglial cells and neurons $\beta$-MCC, an enzyme characteristic of the irreversible part of leucine catabolic pathway. For this purpose $\beta$-MCC was purified from rat liver, and an antiserum was raised against the enzyme. The developed $\beta$-MCC antiserum was subsequently used to examine the presence of $\beta$-MCC in rat astroglia- and neuron-rich primary cultures. $\beta$-MCC staining of astro-
isoenzymes in neural primary cultures from rat brain has also decarboxylates the other two branched-chain acids (Harper et al., 1984). As all catabolites of complex are not specific for acetate and acetyl-CoA. This second step in leucine catabolic pathway should exist in astroglia-rich primary cultures. The initial and still reversible step in leucine degradation is catalyzed in brain by either one of the two BCAT isoenzymes with different subcellular distribution (Roswell and Turner, 1963; Taylor and Jenkins, 1966; Ichihara and Koyama, 1966; Aki et al., 1967). One isoinform is found in mitochondria, whereas the other one is located in the cytosol (Hall et al., 1993). Although BCAT is required for the first step in leucine degradation, its presence is a necessary but not a sufficient piece of evidence for the irreversible catabolism of this amino acid in a cell. The transamination of leucine only reversibly transfers the amino group to α-ketoglutarate, thereby generating glutamate and α-ketoisocaproate. Reamination of the α-keto acids can readily take place (Taylor et al., 1970; Harper et al., 1984), either in the same cells or after export to neighboring cells. Indeed, metabolic studies demonstrated an efficient release of α-ketoisocaproate generated from leucine by cultured astroglial cells (Bixel and Hamprecht, 1995; Yudkoff et al., 1996a). In contrast, the flux of nitrogen from glutamate to leucine in rat cortical synaposomes was demonstrated to be strikingly greater than in the opposite direction (Yudkoff et al., 1996b). It is noteworthy that in neurons and also in astroglial cells the rate of leucine transamination considerably exceeds the rate of the subsequent oxidative decarboxylation (Hutson et al., 1998). The BCKDH, which catalyzes this irreversible multienzyme reaction, initiates the irreversible degradation of α-ketoisocaproate to acetocacetate and acetyl-CoA. This second step in leucine degradation is rate-limiting, even though the BCKDH complex is not specific for α-ketoisocaproate because it also decarboxylates the other two branched-chain α-keto acids (Harper et al., 1984). As all catabolites of α-ketoisocaproate generated in the course of the reaction sequence are CoA derivatives, all enzymes involved must be located in mitochondria. The lack or dysfunction in a cell of one of the enzymes would already result in a pathological condition (Sweetman, 1989). To allocate cellularly the leucine catabolic pathway to acetoacetate in neural cells, it is therefore most likely sufficient to identify cells expressing one enzyme unique to this pathway, such as β-MCC-expressing cells.

Recently a cell-specific expression of the two BCAT isoenzymes in neural primary cultures from rat brain has been observed (Bixel et al., 1996, 1997). Cultured astroglial cells showed strong BCATm immunoreactivity, whereas BCATc was detected in this cell type in varying amounts. BCATc was found in high concentration in cultured neurons and oligodendroglial cells. Based on these findings the authors suggested that the cell-specific isoenzyme expression in neural cells reflects a functional compartmentation of BCAA metabolism in brain. Because cultured astroglial cells readily catalyze leucine (Bixel and Hamprecht, 1995) with the leucine oxidative pathway being located in the mitochondria, it was hypothesized that BCATm is the isoenzyme initiating the breakdown of leucine carbon skeleton in astroglial cells (Bixel et al., 1997). On the other hand, the presence of BCATc in neurons and astroglial cells is compatible with the suggestion of Yudkoff et al. (1996b) that leucine and its cognate α-keto acid may be involved in the intercellular transfer of nitrogen from neurons to astrocytes. Therefore, BCATc most likely serves a function other than shunting leucine into the catabolic pathway, namely, the interconversion of leucine and α-ketoisocaproate in the cytosol of neurons and astroglial cells (Bixel et al., 1997), that forms the enzymatic basis for the intercellular α-ketoisocaproate/leucine cycle (Yudkoff et al., 1996b, 1997; Bixel et al., 1997).

The coexpression of BCAT and β-MCC characterizes cell types with the ability to degrade leucine as "metabolic fuel" in brain energy metabolism. Because cytosolic α-ketoisocaproate passes the inner mitochondrial membrane by simport with one proton (Hutson and Kanellis, 1985), α-keto acids generated by BCATm would be held back in the mitochondria as a consequence of the mitochondrial proton gradient. Thus, once taken up by astroglial cells into the mitochondrial compartment, leucine would be transaminated by the BCATm to α-ketoisocaproate for further oxidative degradation to acetoacetate and acetyl-CoA. The strict colocalization of BCATm and β-MCC in astroglial cells therefore substantiates the hypothesis that mitochondria are the compartment in which BCAA are degraded to acetoacetate. Conversely, the presence of BCATm is likely to indicate the capacity of a cell to subject leucine to terminal degradation. Thus, the hypothesis is inescapable that BCATm should always be accompanied by β-MCC. Because the only function of β-MCC is participation in the irreversible degradation of leucine to acetoacetate, the apparent lack of BCATm in neurons indicates that the transamination of leucine must take place in another compartment, i.e., the neuronal cytosol, which indeed contains an appropriate enzyme, BCATc, or another cell, e.g., the astrocyte. Thus, BCATc appears to be the more versatile isoform because its cytosolic location would allow it to participate in the intercellular α-ketoisocaproate/leucine cycle operating between astrocytes and neurons (Yudkoff et al., 1996b, 1997; Bixel et al., 1997) and to prepare the carbon skeleton of leucine for the subsequent mitochondrial breakdown.

To ensure during activity of glutamatergic neurons that a sufficient amount of α-ketoisocaproate is available...
as amino group acceptor for the nitrogen shuttle, the mitochondrial catabolism of \( \alpha \)-keto acids would have to be strictly regulated. The most likely enzyme for regulation is BCKDH. Besides covalent modification by a phosphorylation/dephosphorylation cycle leading to inhibition or activation of BCKDH, respectively (Harris et al., 1986, 1997), the enzyme complex is allosterically activated by branched-chain \( \alpha \)-keto acids ( Paxton and Harris, 1984 ). In most tissues BCKDH is preferentially found in the phosphorylated, inactive form ( Harris et al., 1986 ). Cytosolic transamination and subsequent release of \( \alpha \)-ketoisocaproate by neurons most likely keep the cytosolic and consequently the mitochondrial \( \alpha \)-keto acid concentration at a low level, at which BCKDH remains in its inactive conformation. In metabolic situations in which the \( \alpha \)-ketoisocaproate concentration in the neuronal cytosol significantly increases, an accelerated uptake of the \( \alpha \)-keto acid into mitochondria could eventually allosterically activate the dehydrogenase complex, leading to oxidative breakdown of \( \alpha \)-ketoisocaproate. Recent studies using neuron-rich primary cultures proved that reactivation of \( \alpha \)-ketoisocaproate significantly exceeds oxidation when its concentration in the culture medium is \( \leq 30 \) \( \mu \)M, whereas \( \alpha \)-ketoisocaproate concentrations \( \geq 100 \) \( \mu \)M resulted in an increase in the rate of oxidation yielding \( \approx 20\% \) of the transamination rate ( Hutson et al., 1998 ). Consequently, the presence of \( \beta \)-MCC in neurons indicates the capacity of these cells to use \( \alpha \)-ketoisocaproate as a fuel molecule. Neurons lacking \( \beta \)-MCC could only partially make use of the energy content of \( \alpha \)-ketoisocaproate, i.e., after its degradation to ketone bodies by neighboring astrocytes. These cells could then export the ketone bodies generated to adjacent neurons ( Bixel and Hamprecht, 1995 ). Neuronal primary cultures have been shown to use ketone bodies for the generation of energy ( Edmond et al., 1987 ). Whether or not all neurons are capable of using ketone bodies for energy production remains to be elucidated. In contrast to neurons, all astroglial cells in a culture examined immunocytochemically appeared to contain \( \beta \)-MCC and are therefore capable of using leucine as a “fuel molecule” for the generation of energy.

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