Branched-chain amino acid metabolism and alanine formation in rat muscles *in vitro*

Mitochondrial–cytosolic interrelationships

Keith SNELL and David A. DUFF

Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

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1. Muscle branched-chain amino acid metabolism is coupled to alanine formation via branched-chain amino acid aminotransferase and alanine aminotransferase, but the subcellular distributions of these and other associated enzymes are uncertain. 2. Recovery of branched-chain aminotransferase in the cytosol fraction after differential centrifugation was shown to be accompanied by leakage of mitochondrial-matrix marker enzymes. By using a differential fractional extraction procedure, most of the branched-chain aminotransferase activity in rat muscle was located in the mitochondrial compartment, whereas alanine aminotransferase was predominantly in the cytosolic compartment. Phosphoenolpyruvate carboxykinase, like aspartate aminotransferase, was approximately equally distributed between these subcellular compartments. 3. This arrangement necessitates a transfer of branched-chain amino nitrogen and carbon from the mitochondria to the cytosol for alanine synthesis *de novo* to occur. In incubations of hemidiaphragms from 48h-starved rats with 3 mM-valine or 3 mM-glutamate, the stimulation of alanine release was inhibited by 69% by 1 mM-aminomethoxybut-3-enoate, a selective inhibitor of aspartate aminotransferase. Leucine-stimulated alanine release was unaffected. 4. These data implicate aspartate aminotransferase in the transfer of amino acid carbon and nitrogen from the mitochondria to the cytosol, and suggest that oxaloacetate, via phosphoenolpyruvate carboxykinase, can serve as an intermediate on the route of pyruvate formation for muscle alanine synthesis.

Branched-chain amino acids are unique in that muscle rather than liver is the major tissue site involved in the initiation of their degradative metabolism (Adibi, 1976; Harper & Zapalowski, 1981; Lund, 1981). The branched-chain amino acids are the most effective precursors for alanine formation in muscle, as a source both of amino nitrogen and perhaps of carbon (Garber *et al.*, 1976; Goldstein & Newsholme, 1976; Snell & Duff, 1977, 1981, 1982). In this regard, they play an important role in glucose homeostasis by determining the availability of alanine as a gluconeogenic precursor (see Snell, 1980). The initial steps in the metabolism of the branched-chain amino acids in muscle are reversible transamination, followed by the irreversible oxidative decarboxylation of the branched-chain oxo acids.

Oxidative decarboxylation is generally considered to be a rate-controlling step in the metabo-

lism and is catalysed by a branched-chain 2-oxo acid dehydrogenase enzyme complex, which appears to be exclusively located in the mitochondrial compartment (Johnson & Connelly, 1972; Van Hinsbergh *et al.*, 1979; Odessey & Goldberg, 1979). The branched-chain amino acid aminotransferase occurs as isoenzymes in animal tissues, and the cytosolic activities can be resolved by DEAE-cellulose chromatography into types I and III isoenzymes (EC 2.6.1.42) and a liver- and leucine-specific type II isoenzyme (EC 2.6.1.6) (Ichihara, 1975; Kadowaki & Knox, 1982). The mitochondrial fraction of a variety of tissues, except brain, contains a single type I isoenzyme which differs from the cytosolic form in certain physicochemical properties, but not in kinetic properties or mobility on DEAE-cellulose (Kadowaki & Knox, 1982). Adult-rat skeletal muscle contains only the type I isoenzyme in

both cytosolic and mitochondrial compartments (Kadowaki & Knox, 1982), but the proportion of total cellular activity distributed between these localizations is controversial. Thus Odessey & Goldberg (1979) report that 70–80% of the aminotransferase activity is present in the soluble fraction of the cell, whereas others have found only about 30–40% in this fraction, with most of the total cellular activity associated with the mitochondrial fraction (Ichihara, 1975; Cappuccino *et al.*, 1978; Kadowaki & Knox, 1982). In addition, Van Hinsbergh *et al.* (1979) showed that [1-¹⁴C]-leucine was effectively decarboxylated by isolated skeletal-muscle mitochondria and, in comparison with rates involving whole homogenates, it is clear that most of the aminotransferase activity must be mitochondrial.

The subcellular localization of branched-chain aminotransferase activity is important in relation to the regulation of further metabolism of the branched-chain 2-oxo acid and to the formation of alanine by muscle (see Snell, 1980). In the present study we have reinvestigated the subcellular distributions of branched-chain aminotransferase and of other enzymes implicated in the pathway of alanine formation in muscle (phosphoenolpyruvate carboxykinase, alanine aminotransferase). Previous workers have all used differential-centrifugation techniques to separate subcellular fractions, whereas we have adopted the alternative approach of fractional extraction (Pette, 1966; Taylor *et al.*, 1978) to disclose intracellular compartments successively and to assign enzyme localizations on the basis of comparison with the behaviour of subcellular 'marker' enzymes. By this method it is clear that, in contrast with the report by Odessey & Goldberg (1979), branched-chain amino acid aminotransferase is predominantly mitochondrial in rat skeletal muscle.

Experimental

Materials

Animals. Male rats of an inbred Wistar albino strain (University of Surrey Animal Unit) were maintained in conditions of 12h light/12h darkness at 21°C and were allowed water and food (Spratt's No. 1 Laboratory diet; Spratts Patent Ltd., Barking, U.K.) *ad libitum* or were deprived of food for 48 h, as indicated in the text. Animals were used at a body wt. of 70–100 g and were killed at 10:00 h.

Chemicals. [1-¹⁴C]Leucine, [1-¹⁴C]valine and NaH¹⁴CO₃ were from Amersham International, Amersham, Bucks., U.K. Enzymes, coenzymes and other biochemicals were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K., or Sigma Chemical Co., Poole, Dorset, U.K. Inorganic chemicals were of analytical grade, obtained

from BDH Chemicals, Poole, Dorset, U.K. 2-Amino-4-methoxy-*trans*-but-3-enoate was generously supplied by Dr. W. E. Scott (Hoffman-La Roche, Nutley, NJ, U.S.A.).

Methods

Isolation and incubation of muscles. Diaphragm (Snell & Duff, 1977), soleus and extensor digitorum longus (Maizels *et al.*, 1977; Frayn & Maycock, 1979) muscles were dissected in this sequence and incubated as described previously. Alanine, lactate and pyruvate were assayed enzymically in HClO₄ extracts of the incubation media (Snell & Duff, 1977).

Subcellular fractionation by differential centrifugation. Subcellular fractionation of muscle homogenates was carried out by the methods described below. Each fraction obtained was sonicated at 4°C (4 × 15 s at 5A, with a Soniprobe 1130/1A, from Dawe Instruments, London W.3, U.K.) before assay for enzyme activities.

Method 1: the method of Odessey & Goldberg (1979) was closely followed. Initial homogenization was with a motor-driven all-glass homogenizer in medium containing 0.25M-sucrose, 50mM-KCl, 5mM-MgCl₂, 5mM-EGTA and 50mM-Tris/HCl, at pH 7.8.

Method 2: the method of Newsholme *et al.* (1972) was followed, with homogenization with a glass homogenizer with a Teflon pestle in medium containing 0.25M-sucrose, 5mM-MgCl₂, 1mM-EDTA and 5mM-Tris/HCl, at pH 8.2.

Method 3: the method of Ernster & Nordenbrand (1967) was followed. Tissue was immersed, minced and rinsed several times in ice-cold 0.15M-KCl, suspended in 0.1M-KCl/1mM-ATP/5mM-MgSO₄/1mM-EDTA/50mM-Tris/HCl, pH 7.4, and homogenized with an all-glass homogenizer.

Method 4: the method of Surholt & Newsholme (1981) was followed, with homogenization with a glass homogenizer with a loose-fitting Teflon pestle in medium containing 300mM-mannitol, 0.1% (w/v) defatted bovine serum albumin, 1mM-EDTA and 20mM-Hepes, at pH 7.4.

Fractional extraction of muscle. The procedure described by Taylor *et al.* (1978) was followed. Muscle tissue was rinsed and minced in medium containing 150mM-KCl, 2% (w/v) defatted bovine serum albumin, 2mM-EGTA and 10mM-Hepes, at pH 7.2. Two portions of minced tissue were used for the preparation of whole homogenates in an all-glass homogenizer in the KCl medium indicated above and supplemented with 2.4mM-sodium deoxycholate, and also in a medium containing 100mM-Tris/HCl, 20mM-mercaptoethanol, 5mM-MgCl₂, 1mM-EDTA, pH 7.4, and supplemented with 2.4mM-sodium deoxycholate. Whole-homogenate enzyme activities were essentially

identical in either of these homogenizing media. A further portion of minced tissue was manually homogenized in a glass homogenizer with a loose-fitting Teflon pestle in 20 vol. of the KCl-based medium indicated above (not supplemented with deoxycholate), and enzyme activities were extracted in three successive steps by stirring and centrifugation of the suspension at 30 000g for 15 min as detailed by Taylor *et al.* (1978). The fourth extraction was carried out by stirring with 100 mM-potassium phosphate, pH 7.2, and after centrifugation the pellet was finally homogenized in medium containing 100 mM-Tris/HCl, 20 mM-2-mercaptoethanol, 5 mM-MgCl₂, 1 mM-EDTA and 2.4 mM-sodium deoxycholate, at pH 7.4, with an all-glass homogenizer. Enzymes were assayed at all steps of the extraction procedure and in the final suspension, as well as in the original whole homogenates. Deoxycholate (2.4 mM) was without effect on any of the enzymes assayed in the present work, and was as effective as sonication in exposing latent mitochondrial enzyme activities in the whole homogenates.

Enzyme assays. Branched-chain amino acid aminotransferase was assayed by using either L-[1-¹⁴C]leucine or L-[1-¹⁴C]valine and measuring ¹⁴CO₂ release from the product after oxidative decarboxylation with H₂O₂ as described by Cappuccino *et al.* (1978). Alanine aminotransferase and aspartate aminotransferase were assayed as described by Segal *et al.* (1962) and Herzfeld & Greengard (1971) respectively. Phosphoenolpyruvate carboxykinase was assayed by the ¹⁴CO₂-incorporation assay (Method A) recommended by Duff & Snell (1982). Glutamate dehydrogenase, lactate dehydrogenase and citrate synthase were assayed by the methods of Williamson *et al.* (1967), Kornberg (1955) and Alp *et al.* (1976) respectively. Succinate dehydrogenase was assayed by a method involving the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and measured at 490 nm (Arrigoni & Singer, 1962). The assay of pyruvate kinase was based on that described by Zammit *et al.* (1978).

All enzyme assays were carried out at 30°C, except for branched-chain amino acid aminotransferase and succinate dehydrogenase assays, which were conducted at 37°C. Activities were calculated as $\mu\text{mol/min per g}$ of muscle tissue.

Results and discussion

Subcellular fractionation by differential centrifugation

By using the procedure described by Newsholme *et al.* (1972) (Method 2, in the Experimental section), the subcellular distribution of branched-

chain amino acid aminotransferase was determined in diaphragm, soleus and extensor-digitorum muscle homogenates with either leucine or valine as amino donor. As noted previously in some other adult tissues (Cappuccino *et al.*, 1978), we found that activities in the three muscle types were very similar with leucine or valine as substrate (results not shown). Whole-homogenate activities in the muscle types were in the proportions 1:0.65:0.52 for diaphragm, soleus and extensor digitorum longus muscles. However, regardless of muscle type and amino acid substrate, 61–76% of the whole-homogenate aminotransferase activity was recovered in the cytosol fraction (in five separate experiments), with an average recovery of 2% of succinate dehydrogenase, the mitochondrial marker enzyme. These results are very similar to those reported by Odessey & Goldberg (1979). Indeed, by using their procedure (Method 1, in the Experimental section), virtually identical recoveries were obtained (results not shown). However, in addition to the mitochondrial-inner-membrane enzyme succinate dehydrogenase, we also assayed the mitochondrial-matrix enzyme glutamate dehydrogenase. In the above experiments this activity was recovered in the cytosol fraction as 56–74% of the whole-homogenate activity. These results suggested that mitochondrial damage and enzyme leakage were occurring during the homogenization and fractionation procedures, presumably attributable to the difficulty associated with homogenizing a tissue containing a large amount of connective tissue. For this reason the cytosolic distribution of branched-chain amino acid aminotransferase as determined above was considered unreliable.

In an effort to improve the subcellular-fractionation procedure, alternative methods involving modifications to the homogenization and fractionation media were explored. The non-sucrose-containing medium used by Ernster & Nordenbrand (1967) (Method 3, in the Experimental section) was based on that devised by Chappell & Perry (1954) to maintain myofibrils in a physical state that allows for easier homogenization. The medium used by Surholt & Newsholme (1981) (Method 4, in the Experimental section) included serum albumin to 'stabilize' mitochondria and improve the integrity of the isolated organelle. With these methods, succinate dehydrogenase was undetectable in the cytosol fraction, but glutamate dehydrogenase was present at 56% (Method 3) or 24% (Method 4) of whole-homogenate activity (averages of four experiments). Citrate synthase, another mitochondrial-matrix marker enzyme, was also recovered as 37% of whole-homogenate activity in the cytosol fraction (Method 4). In our hands neither of these methods improved the

integrity of mitochondrial preparations to a point where we could be confident of any observed sub-cellular distribution of the branched-chain amino acid aminotransferase.

Fractional extraction of extra- and intra-mitochondrial enzymes

In view of the difficulties encountered above with differential-centrifugation procedures, an alternative approach to locating enzyme activities within muscle tissue was used which involved the fractional extraction of tissue minces with aqueous media to disclose intracellular compartments successively and release their associated enzymes (see Taylor *et al.*, 1978). Lactate dehydrogenase and pyruvate kinase were assayed as cytosolic marker enzymes, and glutamate dehydrogenase and citrate synthase were measured as mitochondrial-matrix marker enzymes. The basis of the five fractions obtained is detailed in the Experimental section (see also Taylor *et al.*, 1978), and the fractionation profile for diaphragm muscle is shown in Fig. 1. The pattern for branched-chain amino acid aminotransferase closely follows that for the mitochondrial markers glutamate dehydrogenase and citrate synthase, and was clearly distinguishable from the patterns of the cytosolic enzyme markers. Similar findings were obtained with soleus and extensor digitorum longus muscles (results not shown). This implies a predominantly mitochondrial location for muscle branched-chain amino acid aminotransferase, in contrast with conclusions reached on the basis of differential-centrifugation studies (see above; Odessey & Goldberg, 1979). The present results suggest that the findings of Odessey & Goldberg (1979) were due to mitochondrial damage, undetectable by their reliance on the membrane-bound succinate dehydrogenase as a mitochondrial marker enzyme.

Thus the major part of branched-chain amino acid aminotransferase activity in muscle is located in the mitochondrial compartment, which is also involved in the further metabolism of the oxo acid carbon skeleton (Johnson & Connelly, 1972; Van Hinsbergh *et al.*, 1979). This arrangement, in skeletal muscle at least, obviates the need for a specific mitochondrial carrier or other mechanisms for the transport of branched-chain oxo acids from the cytosol. In muscle from starved rats the further metabolism of the branched-chain oxo acids may lead to the net formation of citric acid-cycle intermediates to provide pyruvate for the synthesis of alanine *de novo* (see Snell, 1980; Snell & Duff, 1981, 1982). The glutamate produced by the transamination of branched-chain amino acids is the amino donor for alanine formation via transamination with pyruvate (Goldstein & Newsholme, 1976; Snell & Duff, 1977; Snell, 1980)

Fig. 1. Fractional extraction of extra- and intra-mitochondrial enzyme activities in rat diaphragm muscle. The values at 30, 60 and 90 min represent extractions with KCl-based medium; those at 120 min and beyond represent extraction with phosphate buffer and the homogenized sediment, respectively. Further experimental details are given in the text. Enzyme activities are expressed as a percentage of the total activity recovered. Each value is the mean of three determinations with separate animals. For all enzymes the total activity recovered (i.e. the sum of the activity released after 120 min extraction and that remaining in the sediment) was between 80 and 110% of the corresponding activity in whole-homogenates prepared in deoxycholate-containing buffer. Whole-homogenate activities ($\mu\text{mol/min per g}$ of tissue) in deoxycholate-containing buffer were: ○, branched-chain (leucine) aminotransferase, 1.15 ± 0.12 ; □, glutamate dehydrogenase, 3.54 ± 0.41 ; △, citrate synthase, 36.3 ± 2.2 ; ●, alanine aminotransferase, 5.86 ± 0.41 ; ▲, lactate dehydrogenase, 319 ± 29 ; ■, pyruvate kinase, 217 ± 2 ; ▽, phosphoenolpyruvate carboxykinase, 0.033 ± 0.005 ; ▼, aspartate aminotransferase, 178 ± 10 ; (means $\pm$ S.E.M. for three experiments).

(see Scheme 1). This latter reaction is catalysed by alanine aminotransferase, which occurs as isoenzyme forms located in the mitochondria and cytosol (Snell & Walker, 1972; De Rosa & Swick, 1975). By using the present fractional extraction

(w/v) homogenates of the muscle were prepared in a Polytron homogenizer in 0.4M-sucrose and kept at 4°C for 30 min with occasional vortex-mixing in the presence of 0.5% Triton X-100 (final concn.) before assay for alanine aminotransferase and aspartate aminotransferase. The latter activity was inhibited by 92–95% and the former by 49–54% under these conditions (in three experiments, results not shown; see also Snell, 1978). This confirms the selectivity of this inhibitor noted by others in liver homogenates (Snell, 1978) and isolated hepatocyte preparations (Smith *et al.*, 1977; Cornell *et al.*, 1984). Aminomethoxybutenoate inhibited valine-stimulated and glutamate-stimulated alanine release by 69%, but was without effect on leucine-stimulated alanine release, in incubations with hemidiaphragms from 48 h-starved rats (Table 1). These results support the hypothesis that mitochondrial oxaloacetate (derived from valine or glutamate metabolism) is transferred to the cytosol in the form of aspartate (via aspartate aminotransferase) and that cytosolic pyruvate for alanine synthesis is then generated in part by the actions of cytosolic aspartate aminotransferase, phosphoenolpyruvate carboxykinase and pyruvate kinase (Scheme 1). The lack of effect on leucine-stimulated alanine formation is due to the fact that metabolism of this amino acid cannot generate oxaloacetate, and the amino nitrogen, derived by mitochondrial transamination, is presumably transferred to the cytosol as glutamate for cytosolic transamination to form alanine. The lack of effect with leucine also attests to the selectivity of aminomethoxybutenoate inhibition of aspartate aminotransferase (cf. alanine aminotransferase). Aminomethoxybutenoate increased the net release of lactate + pyruvate and markedly increased the lactate/pyruvate ratio in the incubation medium

(Table 1). This suggests that glycolysis from residual glycogen is stimulated by this agent, and that the inhibition of aspartate aminotransferase disturbs the operation of the malate–aspartate shuttle, which is involved in NADH re-oxidation through transport of reducing equivalents from the cytosol to the mitochondria (Newsholme & Leech, 1983).

Conclusions

The predominant distribution of branched-chain amino acid aminotransferase activity in the mitochondrial compartment and of alanine aminotransferase activity in the cytosolic compartment revealed in the present study necessitates the transfer of amino nitrogen and of citric acid-cycle carbon from the mitochondria to the cytosol if alanine synthesis *de novo* is to occur. Evidence that this is the case and that it involves the intermediary participation of aspartate has been obtained by using the aspartate aminotransferase inhibitor aminomethoxybutenoate. Other evidence from metabolite determinations in the perfused rat heart has linked an increased formation of alanine with the mitochondrial efflux of aspartate (Safer & Williamson, 1973). Indirectly the present study supports a metabolic route for alanine formation in muscle which involves to some extent the utilization of valine and glutamate carbon for the generation of pyruvate from citric acid-cycle intermediates via phosphoenolpyruvate carboxykinase and pyruvate kinase during starvation (Snell & Duff, 1977, 1982). The extent to which this metabolic route operates *in vivo* during starvation is at present unknown, but it does provide a means for the net conversion of certain amino acids arising from proteolysis into alanine, which, after its

Table 1. *Effect of 1 mM-2-amino-4-methoxy-trans-but-3-enoate on metabolite release in vitro by hemidiaphragms from 48 h-starved rats*

Hemidiaphragms prepared from 48 h-starved rats were preincubated for 30 min in medium without any additions, and then incubated for 2 h in fresh medium containing the additions indicated. Paired incubations were carried out in the presence or absence of 1 mM-2-amino-4-methoxy-trans-but-3-enoate (AMB). Alanine, pyruvate and lactate released into the medium were determined at the end of the incubation and expressed as μmol of metabolite released/2 h per g of tissue. Values are the means $\pm$ S.E.M. for the numbers of experiments shown, and statistical analysis (paired Student's *t* test) of paired values showed significant differences at $**P < 0.01$ or $*P < 0.05$.

Additions to incubation medium	No. of observations	Metabolite release ($\mu\text{mol}/2\text{ h}$ per g of tissue)				[Lactate]/[pyruvate] ratio in medium	
		Alanine		Lactate + pyruvate		Control	+ AMB
		Control	+ AMB	Control	+ AMB		
None	12	1.06 ± 0.12	0.83 ± 0.06	6.54 ± 0.35	$9.27 \pm 0.49^{**}$	3.57	45.4
3 mM-Valine	7	1.93 ± 0.15	$1.10 \pm 0.09^{**}$	5.83 ± 0.42	$9.60 \pm 0.98^{*}$	3.90	79.0
3 mM-Leucine	4	2.07 ± 0.11	1.85 ± 0.12	4.84 ± 0.41	$10.4 \pm 1.02^{*}$	12.8	53.5
3 mM-Glutamate	4	3.25 ± 0.33	$1.50 \pm 0.29^{**}$	8.05 ± 0.95	8.50 ± 0.26	9.19	120

release from muscle, can be used by the liver as a gluconeogenic precursor.

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