The Role of Phosphoenolpyruvate Carboxykinase in Amino Acid Metabolism in Muscle

By ERIC A. NEWSHOLME and THELMA WILLIAMS Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

(Received 15 August 1978)

Starvation or feeding rats on a high-protein diet, valine or isoleucine, but not leucine, increases the activity of muscle phosphoenolpyruvate carboxykinase, but has no effect on NADP⁺-linked malate dehydrogenase. This suggests that muscle phosphoenolpyruvate carboxykinase is involved in oxidation or conversion of some amino acids to alanine.

Since the demonstration of fructose bisphosphatase (EC 3.1.3.11) and PEPCK (EC 4.1.1.32) activities in muscle, the question of the role of these enzymes in this tissue has been raised (Opie & Newsholme, 1967). It has been proposed that these enzymes play a role in glyconeogenesis (i.e. reversal of glycolysis from glycogen) in muscle (Bendall & Taylor, 1970), but lack of detectable activities of pyruvate carboxylase in vertebrate muscle (Crabtree et al., 1972) and lack of correlation between activities of fructose bisphosphatase and PEPCK in a large number of muscles (Newsholme & Crabtree, 1978) suggests that this is unlikely. The role of fructose bisphosphatase in the provision of a substrate cycle between fructose 6-phosphate and fructose bisphosphate to furnish a sensitive mechanism of regulation of glycolytic flux and for a heat-generating mechanism has been discussed in detail elsewhere (Newsholme & Crabtree, 1970, 1973, 1976, 1978; Newsholme, 1976, 1977, 1978). If this latter role for the bisphosphatase is correct, it is unlikely that PEPCK in muscle is functionally related to the bisphosphatase. In a comparative study, Crabtree et al. (1972) observed that PEPCK activities were higher in the more anaerobic vertebrate muscles and they were nondetectable in vertebrate heart and insect flight muscles; these workers were unable to provide an explanation for this difference.

In recent years it has been demonstrated that some amino acids can be oxidized or converted to alanine by muscle. These amino acids include valine, isoleucine, glutamate and aspartate, which are degraded to intermediates of the citric acid cycle and eventually to oxaloacetate. However, for further oxidation or conversion to alanine, the citric acidcycle intermediates must be converted to pyruvate (see Goldstein & Newsholme, 1976). It has been suggested that the role of PEPCK in muscle is to catalyse the conversion of oxaloacetate to phosphoenolpyruvate. The latter is then converted to pyruvate

Abbreviation used: PEPCK, phosphoenolpyruvate carboxykinase.

which could be converted to either acetyl-CoA for oxidation or alanine for release from the muscle. The role of PEPCK in the pathway has received support from the work of Snell & Duff (1977), who demonstrated that, in the isolated diaphragm preparation, mercaptopicolinate, a specific inhibitor of PEPCK, inhibited alanine production from valine and isoleucine but not from leucine. Furthermore, there are marked differences in the properties of this enzyme isolated from oyster muscle, in which it catalyses the conversion of phosphoenolpyruvate to oxaloacetate, compared with the enzyme from other invertebrate and vertebrate muscles, in which flux through this reaction is considered to be in the direction of phosphoenolpyruvate formation (Zammit & Newsholme, 1978). Despite this evidence, it is possible that citric acid-cycle intermediates are converted to pyruvate via the reaction catalysed by the NADP⁺-linked malate dehydrogansae enzyme (EC 1.1.1.40).

In order to obtain further information on the possible role of PEPCK or NADP⁺-linked malate dehydrogenase in amino acid conversion to alanine in muscle, maximum activities of these enzymes have been measured in muscle of various mammals, and the effects of starvation and other conditions that are expected to increase amino acid metabolism in muscle have been investigated. The activities of fructose bisphosphatase have also been measured as a 'control' activity. The results are presented and discussed in this paper.

Materials and Methods

Chemicals, enzymes and animals

These were obtained from sources given previously (Crabtree & Newsholme, 1972; Crabtree *et al.*, 1972), except for the following: cats were obtained from the Department of Pharmacology, University of Oxford [muscle was removed after completion of class experiments which involved anaesthetization with chloralose and intravenous injections of acetylcholine $(4\mu g)$ or eserine $(100\mu g]$; muscle was obtained from human volunteers (male) by biopsy technique at the Institute of Work Physiology, University of Oslo, Norway: dexamethasone was obtained from Sigma Chemical Co., Poole, Dorset, U.K.; streptozotocin was obtained from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Rats were made diabetic by injection of streptozotocin (150mg/kg body wt.) into the tail vein (Blackshear & Alberti, 1974) and used 48 h later. Adrenalectomized rats were obtained from OLAC (1976) Ltd., Blackthorn, Bicester, Oxon OX6 0TP, U.K. Dexamethasone (1.5 mg/kg body wt.) was injected subcutaneously twice daily for 3 days. Rats were made acidotic by addition of NH₄Cl (final concentration 280mm) to the drinking water for 7 days. The high-protein diet contained 80% (by wt.) casein and rats were fed ad libitum. For the leucine, isoleucine and valine diets, rats were meal-fed between 09:00 and 12:00 h with normal Oxoid diet supplemented with 5% (by wt.) of the given amino acid.

Preparation of homogenates and assay of enzyme activities

Homogenization of muscle and assay of PEPCK and fructose 1,6-bisphosphatase were carried out as described by Crabtree *et al.* (1972), and for the malate dehydrogenase as described by Opie & Newsholme (1967), except that, in the PEPCK assay, the coupling enzyme (malate dehydrogenase) was dialysed against homogenization medium for 8h before use with four changes of buffer. This treatment decreased the blank activity probably by lowering $[NH_4^+]$, which activates pyruvate kinase. In some cases, muscle was rapidly frozen and stored in liquid N₂ for several days before use. Preliminary experiments established that this had no effect on the enzyme activities.

Results

The ranges of activities of PEPCK and malate dehydrogenase are, in general, similar in the quadriceps muscle of the mammalian species investigated (0.32–0.86 and 0.14–0.53 μ mol/min per g fresh wt. for PEPCK and the dehydrogenase respectively; see Table 1). In the diaphragm muscle, the activities of PEPCK are, in general, lower than in the quadriceps, but the opposite situation is seen for the malate dehydrogenase (Table 1). Furthermore, PEPCK activities in heart are very low (in confirmation of earlier work: Opie & Newsholme, 1967; Crabtree *et al.*, 1972), except for those in the mouse, whereas the malate dehydrogenase activities are, in general, considerably higher than in the other muscles (Table 1).

Starvation of the rat markedly increased the activity of PEPCK either on a fresh-weight basis or per mg of protein (the activities on a fresh-weight basis are given in Table 1 and those on mg of protein basis are as follows: 1.3 ± 0.41 , 2.4 ± 0.3 , 6.0 ± 0.31 and 8.0 ± 0.24 nmol/min per mg of protein for fed rats and rats starved for 24, 48 and 72h respectively). There was an approximate doubling of the activity every 24h of starvation for the 72h period of study. There was a 50% increase in fructose bisphosphatase after 24h, but this was not apparent after 48 or 72h; the activity of malate dehydrogenase was unaffected (Table 1). A period of 24h of starvation of the mouse caused a marked increase in PEPCK activity, but this was not apparent after 48h starvation (Table 1). However, starvation of the domestic fowl for 48 h caused no change in the activity of PEPCK or the dehydrogenase, whereas that of the bisphosphatase was increased by almost 60%.

Other conditions that are either known or might be expected to increase amino acid metabolism in muscle were investigated in the rat. There was no change in the activities of the three enzymes in muscle in a 48h severely streptozotocin-diabetic rat (Table 1) nor in the quadriceps muscle of adrenalectomized animals or the latter animals given dexamethasone (Table 1). However, feeding on a high-protein diet or a diet supplemented with the individual branchedchain amino acids did produce changes in enzyme activities. The high-protein diet caused an increase in PEPCK activity after 7-14 days, but there was no effect on the activities of either of the other two enzymes (Table 1). Diets containing valine or isoleucine increased the activity of PEPCK in quadricips muscle at both 7 and 14 days from the start of the diet, but there was no effect on the activities of the malate dehydrogenase. All three diets caused an increase in the activity of the bisphosphatase at 7 days (Table 1), but the increases were not statistically significant. Of particular importance (see below) is the observation that a diet supplemented with leucine did not increase the activity of PEPCK; indeed, after 14 days the activity was decreased by 25% (Table 1). The increase in PEPCK activity in the quadriceps muscle caused by starvation (see above) did not occur where the animals were acidotic. However, acidosis itself did not cause any change in **PEPCK** activity (or the other enzyme activities) in the fed animal (Table 1).

Discussion

It has been suggested that the complete oxidation, or conversion of isoleucine and valine (and indeed other amino acids that produce intermediates of the citric acid cycle) to alanine in muscle requires the participation of the reaction catalysed by PEPCK (see Goldstein & Newsholme, 1976; Snell & Duff,

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with numbers of individual animals used given in parentheses. Differences from normals or controls that are statistically significant (Student's *t* test) are shown by $\dagger(P < 0.05)$, $\ast\ast(P < 0.0125)$ and $\ast(P < 0.0025)$.

Enzyme activities (μ mol/min per g of muscle at 25°C)

Animal	Muscle	Condition of animal	PEPCK	Fructose 1,6- bisphosphatase	NADP ⁺ -linked malate de- hydrogenase
Laboratory mouse	Quadriceps Diaphragm Heart	Normal, fed Normal, fed Normal, fed	0.37±0.110 (6) 0.26±0.106 (6) 0.23±0.029 (6)		0.53 ± 0.092 (6) 1.00 ± 0.06 (6) 1.29 ± 0.114 (6)
Laboratory rat	Quadriceps Diaphragm Heart	Normal, fed Normal, fed Normal, fed	0.32±0.030 (6) 0.20±0.023 (6) <0.1		0.41 ± 0.020 (6) 0.78 ± 0.047 (6) 1.8 ± 0.14 (4)
Guinea pig	Quadriceps Diaphragm Heart	Normal, fed Normal, fed Normal, fed	0.36±0.061 (6) 0.18±0.066 (6) 0.14±0.016 (6)	0.14 ± 0.015 (6)	$\begin{array}{c} 0.14 \pm 0.014 \ (6) \\ 0.30 \pm 0.042 \ (6) \\ 0.64 \pm 0.057 \ (6) \end{array}$
Rabbit	Quadriceps Diaphragm Heart	Normal, fed Normal, fed Normal, fed			$\begin{array}{c} 0.17 \pm 0.016 \ (12) \\ 0.33 \pm 0.028 \ (11) \\ 0.26 \pm 0.028 \ (9) \end{array}$
Cat	Quadriceps Diaphragm Heart	Normal, fed Normal, fed Normal, fed	0.62±0.055 (6) 0.49±0.041 (6) 0.09±0.011 (6)	0.78 ± 0.056 (6) 0.60 ± 0.046 (6) 0.03 ± 0.006 (6)	$\begin{array}{c} 0.27 \pm 0.039 \ (6) \\ 0.29 \pm 0.014 \ (6) \\ 1.83 \pm 0.087 \ (6) \end{array}$
Man	Quadriceps	Normal, fed	0.54±0.18 (9)	0.94±0.2 (9)	
Laboratory rat	Quadriceps	Fed, control 24h starvation 48h starvation 72h starvation Fed, control Streptozotocin-diabetic Normal diet 7-day high-protein diet 14-day high-protein diet Fed, acidotic 24h starved, acidotic 48h starved, acidotic	$\begin{array}{c} 2.20 \pm 0.22 (6)^4 \\ 0.54 \pm 0.03 (4) \\ 0.49 \pm 0.02 (7) \\ 0.32 \pm 0.03 (6) \\ 0.33 \pm 0.02 (6) \end{array}$	$\begin{array}{c} 0.37 \pm 0.05 & (6) \\ 0.30 \pm 0.05 & (6) \\ 0.18 \pm 0.01 & (6) \\ 0.24 \pm 0.08 & (4) \\ 0.26 \pm 0.06 & (7) \\ 0.18 \pm 0.02 & (6) \\ 0.18 \pm 0.01 & (6) \\ 0.16 \pm 0.02 & (6) \\ 0.15 \pm 0.032 & (6) \\ 0.12 \pm 0.023 & (6) \end{array}$	$\begin{array}{c} 0.41 \pm 0.02 (6) \\ 0.34 \pm 0.02 (6) \\ 0.31 \pm 0.01 (6) \\ 0.40 \pm 0.01 (6) \\ 0.35 \pm 0.02 (4) \\ 0.43 \pm 0.05 (7) \\ 0.41 \pm 0.02 (6) \\ 0.36 \pm 0.02 (6) \\ 0.36 \pm 0.02 (6) \\ 0.31 \pm 0.02 (6) \\ 0.31 \pm 0.012 (6) \\ 0.38 \pm 0.011 (6) \end{array}$
Rat	Quadriceps	Normal control Adrenalectomized Adrenalectomized given dexamethasone 7-day fed, control 7-day fed, valine 7-day fed, isoleucine 7-day fed, leucine 14-day fed, valine 14-day fed, valine 14-day fed, isoleucine 14-day fed, leucine	$\begin{array}{c} 0.30 \pm 0.018 \ (4)'\\ 0.42 \pm 0.025 \ (4)'\\ 0.18 \pm 0.050 \ (4)\\ 0.23 \pm 0.017 \ (4)\\ 0.37 \pm 0.005 \ (4)'\\ 0.35 \pm 0.021 \ (4)'\end{array}$	$\begin{array}{c} 0.16 \pm 0.03 (6) \\ 0.12 \pm 0.03 (4) \\ 0.19 \pm 0.02 (4) \\ \end{array} \\ \begin{array}{c} 0.20 \pm 0.066 (4) \\ \bullet^{\bullet \bullet} 0.34 \pm 0.077 (4) \\ \bullet^{\bullet} 0.35 \pm 0.090 (4) \\ 0.15 \pm 0.051 (4) \\ \bullet^{\bullet} 0.20 \pm 0.039 (4) \\ \bullet^{\bullet} 0.09 \pm 0.010 (4) \\ \bullet^{\bullet} 0.12 \pm 0.035 (4) \end{array}$	0.44 ± 0.023 (4) 0.47 ± 0.023 (4) 0.36 ± 0.023 (4) 0.42 ± 0.019 (4) 0.30 ± 0.013 (4) 0.37 ± 0.088 (4)
Mouse	Quadriceps	Fed, control 24h starvation 48h starvation	0.37 ± 0.045 (6) 0.51 ± 0.065 (6) 0.34 ± 0.009 (6)		
Domestic fowl	Pectoral	Fed, control 48h starvation	1.34±0.122 (12 1.32±0.191 (12) 0.46±0.053 (12) 0.72±0.118 (12) 0.64±0.060 (14))†0.68±0.062 (12)
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1977). Support for this view is obtained from the present results, which demonstrate that starvation of the rat or mouse, which increases the rate of conversion of branched-chain amino acids to alanine (Odessey et al., 1974), causes marked increases in the activity of muscle PEPCK without any change in that of the NADP+-linked malate dehydrogenase. It has been suggested that some of the newly absorbed dietary amino acids, and especially the branchedchain amino acids, may be metabolized in the muscle rather than the liver (Felig, 1975). If the metabolic pathway for amino acid catabolism involves the PEPCK reaction, then diets with an increased protein or amino acid content might be expected to increase the activity of this enzyme. Thus a high-protein diet does indeed increase the activity of this enzyme. However, of particular importance is the observation that diets supplemented with isoleucine or valine increase markedly the activity of PEPCK, whereas supplementation with leucine has no effect. The metabolism of leucine, unlike that of valine or isoleucine, does not involve the PEPCK reaction (see Snell & Duff, 1977).

The fact that streptozotocin-diabetes increases protein degradation in muscle but does not cause a change in PEPCK activity suggests that other conditions characteristic of the diabetic animal prevent the increase in enzyme activity. Diabetes is characterized by acidosis, and it is noted that in the present work, although acidosis did not change PEPCK activity in the fed animal, it prevented the increase that occurs in muscle during starvation (Table 1).

PEPCK activity is present in skeletal and diaphragm muscle of five mammalian species investigated and, in addition, is present in skeletal muscle of man. It is suggested that the role of this enzyme in muscle is to catalyse a reaction that is part of the metabolic pathway by which certain amino acids are either oxidized or converted to alanine (see Goldstein & Newsholme, 1976). This explanation is consistent with the original observations of Opie & Newsholme (1967) and Crabtree et al. (1972) that there is little or no detectable activity of PEPCK in heart and insect flight muscles and that the activity is lower in aerobic compared with anaerobic muscles. Thus heart and some aerobic muscles are involved in essential physiological functions (e.g. pumping blood, respiration, maintenanance of posture) and it would be expected that these muscles should suffer less protein degradation and amino acid metabolism during starvation (or other conditions) than the less essential and more anaerobic muscles (e.g. the activities of PEPCK in the postural aerobic semitendinosus and the anaerobic adductor magnus muscles of the rabbit are 0.1 and $2.0 \mu mol/min$ per g respectively). Indeed, it has been established that during starvation heart muscle suffers considerably less protein degradation than does skeletal muscle (Jefferson *et al.*, 1974).

If PEPCK is indeed part of a metabolic pathway for converting certain amino acids into alanine, then it is possible that a deficiency of this enzyme specifically in muscle of human subjects could account for the hypoalanaemia that is associated with the condition of ketotic hypoglycaemia in children (Pagliara *et al.*, 1972).

We thank Professor R. R. Porter, F.R.S., for his interest and encouragement and Dr. L. Hermansen for removal, via the biopsy technique, of small quantities of human muscle.

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