The Activities of Pyruvate Carboxylase, Phosphoenolpyruvate Carboxylase and Fructose Diphosphatase in Muscles from Vertebrates and Invertebrates

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1. The activities of pyruvate carboxylase, phosphoenolpyruvate carboxylase and fructose diphosphatase in crude homogenates of vertebrate and invertebrate muscles are reported. 2. Pyruvate carboxylase activity was present in all insect flight muscles that were investigated: in homogenates of bumble-bee flight muscle the activity was inhibited by ADP and activated by acetyl-CoA, and it was distributed mainly in the mitochondrial fraction. This is the first demonstration of pyruvate carboxylase activity in muscle. However, the activity appears to be restricted to insect flight muscle, since it was not found in other invertebrate or vertebrate muscles. 3. Since the three enzymes were never found together in the same muscle, it is concluded that these enzymes cannot provide a pathway for the synthesis of glycogen from lactate or pyruvate in muscle. Other roles for these enzymes in muscle are suggested. In particular, pyruvate carboxylase may be present in insect flight muscle for the provision of oxaloacetate to support the large increase in activity of the tricarboxylic acid cycle which occurs when an insect takes flight.

The enzymes pyruvate carboxylase, phosphoenolpyruvate carboxylase (formerly carboxykinase) and fructose diphosphatase (EC 6.4.1.1, 4.1.1.32 and 3.1.3.11, respectively) catalyse reactions which are important for the operation of gluconeogenesis in tissues such as liver and kidney cortex (for review see Newsholme & Gevers, 1967). The detection of fructose diphosphatase and phosphoenolpyruvate carboxylase activities in some skeletal muscles (Krebs & Woodford, 1965; Opie & Newsholme, 1967) and the demonstration of glycogen synthesis from lactate in frog sartorius muscle (Bendall & Taylor, 1970) have raised the problem of the enzymology of the pathway for carbohydrate synthesis in muscle. In a reinvestigation of this problem the activities of these three enzymes in a variety of muscles have been measured and the results are reported in the present paper. In particular, a simple and specific assay for pyruvate carboxylase activity (Martin & Denton, 1970) has been used to provide the first unequivocal evidence for the presence of this enzyme in muscle.

Materials and Methods

Chemicals, commercial enzymes and animals

These were obtained from sources given previously (see Crabtree & Newsholme, 1972) except for the following: acetyl-CoA and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Preparation of muscle homogenates

Freshly dissected muscles were homogenized, by using ground-glass homogenizers, in 5–10 vol. of an extraction medium consisting of 100 mM-tris-HCl buffer, 1 mM-EDTA, 5 mM-MgCl₂ and 20 mM-2-mercaptoethanol at a final pH of 7.5. Preliminary experiments with homogenates of bumble-bee, water-bug and locust flight muscle and with homogenates of rat heart and frog sartorius muscle established that the activities of pyruvate carboxylase and phosphoenolpyruvate carboxylase were not increased by sonicating the extracts. Homogenates were assayed for activity within 15 min after preparation unless otherwise stated.

Assay of enzyme activities

Pyruvate carboxylase activity was assayed by two methods. First, the formation of oxaloacetate was measured by its reaction with acetyl-CoA, in the presence of citrate synthase, to form citrate and CoA; the CoA then reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) to form a compound which absorbs light at 412 nm (Martin & Denton, 1970). The assay medium contained 50 mM-tris-HCl buffer, 0.2 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 10 mM-MgSO₄, 2.5 mM-ATP, 5 mM-creatine phosphate, 0.75 mM-acetyl-CoA, 10 mM-KHCO₃ and 10 mM-pyruvate at a final pH of 7.5. Immediately before the assay 320 µg of creatine kinase and 5 µg of citrate synthase were added to 2 ml of assay medium in a cuvette. The

Table 1. *Activities of pyruvate carboxylase, phosphoenolpyruvate carboxylase and fructose diphosphatase in muscles*

Enzyme activities were measured and the activities are presented as means, with the ranges of activities and the numbers of animals or separate pools of muscle from a larger number of animals given in parentheses. For further details see the Materials and Methods section. The values <0.2 for pyruvate carboxylase, <0.1 for phosphoenolpyruvate carboxylase and <0.05 for fructose diphosphatase mean that the activity (if present) was below the limit of detectability of the assays.

Animal	Muscle	Enzyme activities ($\mu\text{mol/min per g}$)		
		Pyruvate carboxylase	Phosphoenolpyruvate carboxylase	Fructose diphosphatase
Crustacea				
Crab (<i>Cancer pagurus</i>)	Claw	<0.2 (2)	—	0.4 (0.3–0.5) (3)
Mollusca				
Sea mussel (<i>Mytilus edulis</i>)	Posterior adductor	—	2.6 (2.1–3) (3)	0.4 (0.2–0.5) (3)
Orthoptera				
Locust (<i>Locusta migratoria</i>)	Flight	1.7 (1.3–1.8) (3)	<0.1 (4)	0.4 (0.3–0.6) (3)
(<i>Schistocerca gregaria</i>)	Flight	1.5 (1.4–1.6) (4)	<0.1 (3)	0.9*
(<i>Schistocerca gregaria</i>)	Hindleg femoral	<0.2 (4)	0.6 (0.5–0.7) (4)	1.3 (1.2–1.5) (3)
Dictyoptera				
Cockroach (<i>Periplaneta americana</i>)	Flight	0.6 (0.5–0.9) (3)	<0.1 (4)	1.2*
	Coxal (white)	<0.2 (3)	0.9 (0.7–1.2) (4)	0.4 (0.3–0.5) (3)
Hemiptera				
Waterbug (<i>Lethocerus cordofanus</i>)	Flight	2.1 (1.7–2.7) (3)	<0.1 (3)	1.5 (1.1–2) (5)
	Foreleg femoral	<0.2 (2)	0.7 (0.3–1.1) (4)	<0.05 (3)
Hymenoptera				
Wasp (<i>Vespa vulgaris</i>)	Flight	12 (11–13) (3)	<0.1 (3)	<0.05 (4)
Honey bee (<i>Apis mellifera</i>)	Flight	4.8 (4.1–5) (3)	<0.1 (4)	<0.05†
Bumble bee (<i>Bombus pratorum</i>)	Flight	19 (18–19) (4)	<0.1 (3)	73†
(<i>Bombus agrorum</i>)	Flight	17 (17–18) (3)	<0.1 (3)	38†
Queen bumble bee (<i>Bombus terrestris</i>)	Flight	12 (11–12) (3)	—	18†
Cuckoo bee (<i>Psithyrus vestalis</i>)	Flight	18 (17–18) (2)	—	2.2†
Lepidoptera				
Buff-tip moth (<i>Phalera bucephala</i>)	Flight	5.2 (4.9–5.5) (2)	<0.1 (2)	<0.05 (4)
Herald moth (<i>Scoliopteryx libatrix</i>)	Flight	4.7 (4.2–5.2) (2)	—	<0.05 (3)
Yellow-underwing moth (<i>Noctua pronuba</i>)	Flight	2.3 (2.1–2.5) (2)	<0.1 (2)	<0.05 (2)
Diptera				
Fleshfly (<i>Sarcophaga barbata</i>)	Flight	—	<0.1 (4)	1.3*
Blowfly (<i>Calliphora vomitoria</i>)	Flight	10 (9–11) (3)	<0.1 (2)	—
(<i>Calliphora erythrocephala</i>)	Flight	—	<0.1 (2)	0.9 (0.9, 0.9) (2)
Pisces				
Dogfish (<i>Scylliorhinus canicula</i>)	Red	<0.2 (2)	0.4 (0.3–0.5) (3)	0.2 (0.2–0.3) (4)
	White	<0.2 (2)	0.8 (0.6–0.9) (3)	0.5 (0.3–0.7) (4)

Amphibia	Frog (<i>Rana temporaria</i>)	Aves	Sartorius	<0.2 (3)	1.1 (0.8-1.2) (4)	0.6*
Pectoral	Sparrow (<i>Passer domesticus</i>)	Pigeon (<i>Columba livia</i>)	Pectoral	<0.2 (2)	—	—
			Pectoral	<0.2 (3)	0.5 (0.4-0.6) (4)	0.5 (0.5-0.6) (3)
			Heart	<0.2 (3)	<0.05†	<0.05†
			Pectoral	—	1.2 (0.8-1.8) (4)	0.6 (0.3-0.7) (3)
			Pectoral	<0.2 (2)	4.2 (3.9-6) (4)	4.4*
Mammalia	Rat	Mouse	Heart	<0.2 (2)	—	—
			Quadriceps	<0.2 (3)	<0.1 (3)	<0.05†
			Heart	<0.2 (3)	0.5 (0.5-0.6) (3)	0.3 (0.2-0.3) (3)
			Semitendinosus	<0.2 (3)	<0.1 (3)	<0.05 (4)
			Adductor magnus	<0.2 (3)	2 (1.7-3) (3)	0.9 (0.5-1.1) (4)

* Value from Newsholme & Crabtree (1970).

† Value from Newsholme *et al.* (1972).

‡ Value from Opie & Newsholme (1967).

maximum volume of tissue extract added to the cuvette was 5 μ l, to ensure that the 5,5'-dithiobis-(2-nitrobenzoic acid) was not completely removed by its reaction with mercaptoethanol, which is present in the extraction medium. The rate of increase in E_{412} was measured. Controls, from which pyruvate was omitted, were used to correct for the increase in E_{412} caused by hydrolysis of acetyl-CoA in the presence of extract. Secondly, the formation of oxaloacetate was measured as the rate of oxidation of NADH in the presence of malate dehydrogenase. This method was possible only with extracts of insect flight muscles, which possess very low activities of lactate dehydrogenase, so that interference by the oxidation of NADH in the presence of pyruvate alone is minimal. The assay medium contained 50mm-tris-HCl buffer, 10mm-MgSO₄, 2.5mm-ATP, 5mm-creatine phosphate, 10mm-KHCO₃, 0.17mm-NADH, 1mm-KCN and 10mm-pyruvate at a final pH of 7.5. Immediately before the assay 320 μ g of creatine kinase and 5 μ g of malate dehydrogenase were added to 2ml of assay medium in a cuvette. Assays were initiated by the addition of 2-10 μ l of extract and the rate of oxidation of NADH was measured at 340nm. Controls, from which ATP and the ATP-regenerating system were omitted, measured the NADH oxidation caused by lactate dehydrogenase activity.

Phosphoenolpyruvate carboxylase activity was assayed as described by Opie & Newsholme (1967). The replacement of IDP in the assay medium by an equal concentration of GDP or ADP produced no detectable activity of this enzyme in the insect flight muscle homogenates that were tested.

Fructose diphosphatase activity was assayed as described by Newsholme & Crabtree (1970).

All enzyme activities were determined at 25°C with a Gilford recording spectrophotometer (model 240).

Expression of results

All enzyme activities are expressed as μ mol of product formed/min per g fresh wt. of muscle at 25°C. The values reported represent the means of a number of determinations (with a number of animals or separate pools of muscle from a larger number of animals) and this number together with the range of activities is given in parentheses in Table 1. Since a systematic study of factors such as season, diet, size, age and sex of animals was not attempted in this work, precise quantitative considerations based on the results must be made with caution.

Results

Phosphoenolpyruvate carboxylase activity

Phosphoenolpyruvate carboxylase activity was not detected in homogenates of any of the insect flight

Table 2. *Effects of ADP and acetyl-CoA on the activity of pyruvate carboxylase from the flight muscle of the bumble-bee*

The species of bumble-bee was *B. agrorum*. Homogenates were prepared as described in the Materials and Methods section except that when the effect of acetyl-CoA was investigated the homogenate was passed through a column of Sephadex G-25 that had been equilibrated with extraction medium. When the effect of ADP was tested the enzyme activity was assayed with citrate synthase and 5,5'-dithiobis-(2-nitrobenzoic acid), with the omission of the creatine phosphate plus creatine kinase. When the effect of acetyl-CoA was tested the enzyme activity was assayed with malate dehydrogenase and NADH. This experiment is representative of three similar experiments.

ADP concn. (mM)	Enzyme activity ($\mu\text{mol/min per g}$)	Acetyl-CoA concn. (mM)	Enzyme activity ($\mu\text{mol/min per g}$)
0	12.5	0	1.4
1.0	10.6	0.04	3.8
2.0	8.8	0.18	11.0
4.0	5.0	0.72	11.0
8.0	3.3		

muscles investigated, although it was detected in homogenates of the leg muscles (Table 1). The previous work of Opie & Newsholme (1967) was extended to show that phosphoenolpyruvate carboxylase activity is present in a number of vertebrate muscles: the highest activity observed was $4.2 \mu\text{mol/min per g}$ (pheasant pectoral muscle).

Fructose diphosphatase activity

The previous work of Opie & Newsholme (1967) and Newsholme & Crabtree (1970) was extended and shows that fructose diphosphatase activity is present in many vertebrate and invertebrate muscles. However, the activity of this enzyme was not detectable in some insect flight muscles (notably some species of Lepidoptera and Hymenoptera, see Table 1), and from previous work it is known not to be detectable in mammalian heart muscle, smooth muscle and semitendinosus muscle of the rabbit (Krebs & Woodford, 1965; Opie & Newsholme, 1967).

Pyruvate carboxylase activity

Activities of pyruvate carboxylase were found in extracts of all the insect flight muscles that were tested (Table 1). The highest activity ($19 \mu\text{mol/min per g}$) was observed in extracts of *Bombus pratorum* flight muscle and this value is approximately fourfold that reported for the enzyme from mammalian or avian liver (see Scrutton & Utter, 1968). The activities of pyruvate carboxylase in insect flight muscle extracts were similar whether they were assayed by measuring NADH oxidation with malate dehydrogenase or by measuring the increase in E_{412} in the presence of acetyl-CoA, 5,5'-dithiobis-(2-nitrobenzoic acid) and citrate synthase. With either

Table 3. *Intracellular distribution of pyruvate carboxylase activity in the flight muscles of the bumble-bee*

The species of bumble-bee was *B. terrestris* and the cell fractions were prepared at pH 8.2, as described by Newsholme *et al.* (1972). Preliminary experiments established that these extraction conditions were not detrimental to the enzyme activity. The breakage of mitochondria, estimated as described by Newsholme *et al.* (1972), was approximately 15%, and the activity of pyruvate carboxylase in the initial homogenate was $12 \mu\text{mol/min per g}$ at 25°C . The experiment is representative of three similar experiments.

Cell fraction	Pyruvate carboxylase activity as a percentage of the activity of the initial homogenate
Cell debris	6
Mitochondria	83
Sarcoplasmic reticulum	7
Soluble supernatant	11

assay system the activity depended upon the simultaneous presence of both pyruvate and ATP. In the case of muscle tissue, pyruvate carboxylase activity appears to be restricted to the insect flight muscles, since it was not detected in insect leg muscles or other invertebrate or vertebrate muscles (Table 1).

The activity of pyruvate carboxylase from bumble-bee flight muscle was inhibited by ADP and activated by low concentrations of acetyl-CoA (Table 2). The K_m value for pyruvate (in the presence of 2.5 mM -ATP) was approx. 0.7 mM . These kinetic properties are similar to those of the enzyme from avian liver (Keech & Utter, 1963). Fractionation of bumble-bee flight muscle homogenates indicated that at least

80–90% of the pyruvate carboxylase activity may be located in the mitochondria (Table 3).

Discussion

Extracts of insect flight muscles converted pyruvate into oxaloacetate and this conversion depended upon the presence of ATP. These results provide strong evidence for the presence of the enzyme pyruvate carboxylase in insect flight muscles. This conclusion is reinforced by the kinetic properties of the conversion of pyruvate into oxaloacetate (Table 2): it is activated by acetyl-CoA and inhibited by ADP, and these properties are similar to those of the enzyme from avian liver. The results of the present investigation establish that yet another gluconeogenic enzyme occurs in muscle. Consequently, the three enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxylase and fructose diphosphatase) which are needed to bypass the irreversible reactions of glycolysis (and thus permit carbohydrate synthesis from lactate and pyruvate), are all found in muscle tissue. However, the other results of the present investigation show that detectable activities of all three enzymes do not occur together in any one type of muscle. All insect flight muscles so far investigated possess pyruvate carboxylase activity, some insect flight muscles also possess fructose diphosphatase activity, but no insect flight muscle possesses phosphoenolpyruvate carboxylase activity (Table 1); also, some vertebrate and invertebrate muscles possess activities of both fructose diphosphatase and phosphoenolpyruvate carboxylase, but there is no detectable activity of pyruvate carboxylase (Table 1). Further, some muscles (notably mammalian and avian heart muscle) possess no detectable activities of any of the three enzymes. The distribution of these enzymes in muscle is not consistent with a role in carbohydrate synthesis from lactate or other substrates.

In vertebrate white muscles, the enzyme NADP-dependent malate dehydrogenase (formerly 'malic' enzyme) is present in addition to fructose diphosphatase and phosphoenolpyruvate carboxylase (Opie & Newsholme, 1967), so that a conversion of lactate into glycogen is theoretically possible. Bendall & Taylor (1970) demonstrated with frog sartorius muscle that glycogen can be synthesized from lactate. However, the physiological significance of this conversion is questionable, since it occurred in the absence of glucose and a high concentration of lactate was maintained in the medium for several hours.

If the three enzymes investigated in the present work are not involved in carbohydrate synthesis in muscle the question arises as to their metabolic role in this tissue, particularly in relation to their distribution in different muscles. The role of fructose

diphosphatase in muscle has been discussed in relation to the regulation of glycolysis (Newsholme & Crabtree, 1970). In bumble-bee flight muscle the activity of fructose diphosphatase is very high and has been discussed in relation to the production of heat (Newsholme *et al.*, 1972).

The role of phosphoenolpyruvate carboxylase in vertebrate muscles is still uncertain. In some invertebrate tissues this enzyme is involved in the conversion of carbohydrate into succinate under anaerobic conditions (see Awapara & Simpson, 1967; Pritchard & Schofield, 1968). This pathway produces one extra mol of ATP/mol of glucose degraded, compared with lactate formation. The activity of phosphoenolpyruvate carboxylase in the adductor muscle of *Mytilus* is similar to that of phosphofructokinase (see Crabtree & Newsholme, 1972) and this suggests that the formation of succinate could be quantitatively important in this muscle during mechanical activity. However, in vertebrate muscles the activity of phosphofructokinase is 10–30-fold that of phosphoenolpyruvate carboxylase in the same type of muscle (see Table 1 and Crabtree & Newsholme, 1972), so that it is unlikely that a succinate-producing pathway is of quantitative importance for ATP production during mechanical activity. It is possible, however, that such a pathway is of importance in resting vertebrate muscle, when the glycolytic rate is very low.

Any hypothesis to explain the presence of pyruvate carboxylase in muscle must take into account its apparent restriction to insect flight muscles. A main characteristic of such muscles is the extremely high oxidative metabolic rate which is necessary to support the mechanical activity of insect flight (see Sacktor, 1965; Weis-Fogh, 1967). Consequently, in insect flight muscles, the activities of the enzymes of the tricarboxylic acid cycle are very high (Beenackers, 1969) and are much greater than those in other types of muscle (Crabtree, 1969). However, at rest the metabolic rate and hence the activity of the tricarboxylic acid cycle in insect flight muscle may be decreased to 2% of that during flight (see Sacktor, 1965) so that, when the insect takes flight, the activity of the cycle may eventually be increased 50-fold. To accommodate this large increase in the rate of the tricarboxylic acid cycle, it is likely that oxaloacetate must be provided from reactions outside the cycle. It is suggested that pyruvate is the source of this oxaloacetate and that the enzyme pyruvate carboxylase is present in insect flight muscle to provide the oxaloacetate when required. At the present time the mechanism for the regulation of pyruvate carboxylase activity in relation to the activity of the tricarboxylic acid cycle is uncertain, but one possibility is control by changes in the mitochondrial concentration of acetyl-CoA. This proposed role for pyruvate carboxylase in muscle is supported by the work of Hansford (1972), who has shown that mitochondria

isolated from a blowfly oxidize pyruvate in the presence of ATP and bicarbonate, but not in the absence of these compounds. It must be stressed that this role for pyruvate carboxylase in the provision of oxaloacetate for the synthesis of tricarboxylic acid cycle intermediates is not new: it has been proposed for the enzyme in bacteria and yeast (see Kornberg, 1966). Also, the amino acid proline has been suggested as a source of oxaloacetate for the tricarboxylic acid cycle in insect flight muscles (Sacktor & Childress, 1967).

The absence of pyruvate carboxylase activity from other aerobic muscles (notably heart muscle and avian pectoral muscle) suggests either that the maximum activity and/or the variation in the rate of the tricarboxylic acid cycle is much smaller in these muscles than in insect flight muscles, or that a separate mechanism for the production of oxaloacetate is present in these muscles.

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