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PARTIAL PURIFICATION AND SOME PROPERTIES OF PYRUVATE CARBOXYLASE FROM THE FLIGHT MUSCLE OF THE LOCUST *(SCHISTOCERCA GREGARIA)*

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Summary

A procedure is described for the partial purification of pyruvate carboxylase (pyruvate:CO_2 ligase (ADP-forming), EC 6.4.1.1) from the flight muscle of the locust (*Schistocerca gregaria*). Characterisation of the kinetic properties of this enzyme indicates that it is activated by acetyl-CoA, is insensitive to inhibition by di- and tricarboxylic acids and exhibits an apparent K_m for HCO_3^- (16 mM) which differs by an order of magnitude from that observed for other pyruvate carboxylases. It is suggested that activation of this locust flight muscle pyruvate carboxylase during the rest → flight transition may result from increases in the concentrations of pyruvate and HCO_3^- under these conditions.

In mammals, birds and amphibians pyruvate carboxylase (pyruvate:CO_2 ligase(ADP-forming), EC 6.4.1.1) is present in tissues such as liver, kidney, adipose tissue, brain and adrenal gland [1, 2]; and is believed to be responsible for replenishment of the oxaloacetate pool when this metabolite is withdrawn for participation in biosynthetic pathways such as lipogenesis and steroidogenesis [3]. Such a role may be termed external anaplerotic since the need for replenishment results from a demand for oxaloacetate originating outside the tricarboxylic acid cycle. Pyruvate carboxylase activity has not been detected in muscle tissue in vertebrates with the exception of the recent observation that this enzyme is present at significant levels in rat and frog heart [4]. In contrast, high levels of pyruvate carboxylase are present in the flight muscles of insects from the orders Hymenoptera, Lepidoptera, Diptera and Orthoptera [5]. Since no external anaplerotic role for the enzyme ap-

pears to exist in the flight muscle, it has been suggested that synthesis of oxaloacetate by pyruvate carboxylase may provide a mechanism for increasing the levels of tricarboxylic acid cycle intermediates hence permitting a higher rate of ATP synthesis. This process is described as internal anaplerotic since it is suggested that the oxaloacetate generated stays within the tricarboxylic acid cycle [6]. No studies have been reported for pyruvate carboxylase either from an insect tissue or from any type of muscle. Hence we have partially purified and examined the enzyme from the flight muscle of the locust (*Schistocerca gregaria*), focussing especially on those properties which might be relevant to the proposed internal anaplerotic role of this enzyme.

Flight muscle extracts were prepared by homogenising freshly dissected muscle in 10 vols. of 0.3 M sucrose containing 1 mM EDTA and 5 μM phenylmethylsulphonyl fluoride in a ground glass homogeniser at 2°C. For partial purification of flight muscle pyruvate carboxylase, flight muscle (3.6 g) from 20 adult locusts was frozen in liquid N_2 and powdered in a percussion mortar pre-cooled in liquid N_2. The powder was extracted at 2°C with 31 ml 0.25 M sucrose containing 1 mM EDTA and 7.5 μM phenylmethylsulfonyl fluoride. Particulate matter was then removed by centrifugation at 26 000 × *g* for 30 min at 2°C. Lipid was removed from the supernatant by filtration through Whatman No. 1 filter paper. Solid $(NH_4)_2SO_4$ (2.6 g/10ml) (42%) was added to the clarified supernatant fraction and the pH maintained at or above 7.0 by addition of 0.4 M Tris base. The suspension was stirred for 10 min at 15°C and the precipitate which contained minimal pyruvate carboxylase activity was removed by centrifugation at 17 000 × *g* for 15 min at the same temperature. Pyruvate carboxylase was precipitated from the supernatant fraction by addition of solid $(NH_4)_2SO_4$ (1.0 g/10ml) (52%). The precipitate was collected as described above, resuspended in a minimal volume of 1.5 M sucrose containing 0.05 M sodium phosphate (pH 7.0), 1 mM EDTA and 5 μM phenylmethylsulphonyl fluoride, and was stored frozen at −5°C. In a representative purification 75% of the pyruvate carboxylase content of locust flight muscle (3.7 units/g wet weight) was extracted from the frozen precipitate and 48% of the activity in this extract was collected in the 42—52% $(NH_4)_2SO_4$ precipitate, which was not severely contaminated with lactate dehydrogenase. The specific activity of pyruvate carboxylase in the 42—52% $(NH_4)_2SO_4$ precipitate was in the range 1—2 U/mg protein, indicating that these preparations are 5—10% pure based on an assumed maximal specific activity of 20 U/mg [7]. The $(NH_4)_2SO_4$ fractionation should be performed at 15°C since locust flight muscle pyruvate carboxylase is inactivated on incubation at 2°C in the presence of high concentrations of $(NH_4)_2SO_4$.

Pyruvate carboxylase activity was assayed in the presence of citrate synthetase and 5,5'-dithiobis(2-nitrobenzoate) [8] in muscle homogenates and during purification); or in the presence of malate dehydrogenase and NADH [9] (for the partially purified enzyme). Protein was measured by the Biuret method. $(NH_4)_2SO_4$ was removed from commercial malate dehydrogenase preparations by dialysis against 2 × 400 ml 0.01 M Tris/Cl (pH 7.5) containing 2 mM EDTA. Acetyl-CoA was prepared as described by Simon and Shemin [10].

Initial experiments demonstrated that locust flight muscle pyruvate carboxylase shows the typical substrate requirements for this enzyme, and also exhibits no significant catalytic activity in the absence of acetyl-CoA. The apparent K_m values observed for pyruvate, $Mg{\cdot}ATP^{2-}$ and HCO_3^- are given in Table I. For comparison apparent K_m values obtained for chicken liver and rat kidney pyruvate carboxylases, which are generally characteristic of those observed for the vertebrate liver and kidney enzymes, and for a partially purified preparation of pyruvate carboxylase from *Tenebrio molitor* are also given in Table I. All four enzymes show similar apparent K_m values for pyruvate, but the locust flight muscle pyruvate carboxylase exhibits a less favourable apparent K_m for HCO_3^- and, to a lesser extent, for $Mg{\cdot}ATP^{2-}$.

Examination of the properties of activation of locust flight muscle pyruvate carboxylase by acetyl-CoA (Table II) indicates that the values obtained for apparent K_A and n_H are similar to those observed for the enzymes from *T. molitor*, rat liver and rat kidney. Both the apparent K and n_H for activation of locust flight muscle pyruvate carboxylase by acetyl-CoA decrease with increasing pH over the range examined (6.9—7.7). The presence of SO_4^{2-} causes an increase in n_H for acetyl-CoA without a significant change in apparent K_a (Table II) as well as a decrease in apparent V of the locust flight muscle enzyme. This contrasts with the effect of this anion on chicken liver pyruvate carboxylase where it acts as a competitive inhibitor with respect to acetyl-CoA [13]. In addition, the apparent K_A for activation of locust flight muscle pyruvate carboxylase by acetyl-CoA is a function of pyruvate concentration. This is shown in Fig. 1 for data obtained at pH 6.9 and 7.4. The value of n_H shows no significant variation with pyruvate concentration at either pH. These effects are qualitatively similar to those reported previously [14] for rat liver pyruvate carboxylase, but occur over a much lower range of pyruvate concentration (0—0.1 mM).

No metabolites other than acetyl-CoA have yet been found which modulate catalysis by locust flight muscle pyruvate carboxylase. In particular, addition of L-glutamate, L-aspartate, citrate and α-oxoglutarate at concentrations up to 5 mM have no effect. In contrast, various other pyruvate

TABLE I

APPARENT MICHAELIS CONSTANT OBSERVED FOR INTERACTION OF SUBSTRATES WITH LOCUST FLIGHT MUSCLE PYRUVATE CARBOXYLASE

Substrate	Apparent K_m(mM) observed for pyruvate carboxylase from:			
	Locust flight muscle	*T. molitor*	Chicken liver*	Rat kidney
Pyruvate	0.25	0.27	0.39	0.24
$Mg{\cdot}ATP^{2-}$	0.32	0.14	0.08	0.19
HCO_3^-	16.1	1.5	1.2	2.7

*Taken from ref. 11.

carboxylases are inhibited in the presence of these di- and tricarboxylic acids [1, 14]. In the case of the partially purified preparation of pyruvate carboxylase from *T. molitor*, inhibition is observed in the presence of 5 mM citrate or L-glutamate but α-oxoglutarate and L-aspartate are ineffective (Scrutton, M.C. and White, M.D., unpublished data).

The data presented indicate that the properties of locust flight muscle pyruvate carboxylase differ most strikingly from those of the enzymes obtained from vertebrate liver and *T. molitor* in the values observed for the apparent K_m for HCO_3^-, and, to a lesser extent, for $Mg \cdot ATP^{2-}$ (Table I). The properties of activation of the locust flight muscle enzyme by acetyl-CoA appear generally similar to those observed for mammalian liver pyruvate carboxylases (Table II, Fig. 1). No inhibitory modifiers have yet been described for this enzyme. The differences observed in the apparent Michaelis constant for HCO_3^- is striking since for most other pyruvate carboxylases studied similar values are observed for the kinetic constants for the substrates while the nature and properties of the modulation of catalytic activity by effectors varies widely [1]. It is tempting therefore to suggest that the mitochondrial HCO_3^- concentration might play a role in the regulation of oxaloacetate synthesis in locust flight muscle. Mitochondrial HCO_3^- levels would be expected to increase as a consequence of the increased rate of respiration which accompanies the rest → flight transition. This suggestion is supported by the recent observation that trehalase in the haemolymph of *Periplaneta americana* is greatly activated by HCO_3^- indicating that the rate of provision of energy substrate may also be enhanced by the increase in the rate of respiration [15].

Measurements of metabolites relevant to the pyruvate carboxylase

TABLE II

PROPERTIES OF ACTIVATION OF LOCUST FLIGHT MUSCLE PYRUVATE CARBOXYLASE BY ACETYL-CoA

Initial rates of oxaloacetate synthesis were measured as a function of acetyl-CoA concentration in the presence of malate dehydrogenase and NADH as described with Methods. The apparent V was obtained by plotting reciprocal initial velocity against reciprocal [acetyl-CoA]^2, and the data were then analysed according to the Hill equation to give apparent K_A ($[S]_{0.5}$) and n_H, the Hill coefficient. The values for the rat liver and kidney pyruvate carboxylases were obtained in the presence of 5 mM pyruvate.

Source of Enzyme (and pH of assay)	Apparent K_a (μM)	n_H
Locust flight muscle		
pH 7.4*	31 (25)	2.3 (3.3)
pH 7.7	23	2.1
T. molitor pH 7.8	13	2.1
Rat liver pH 7.8**	20	2.0
Rat kidney pH 7.8	31	2.1

*The values shown in parentheses were obtained in the presence of 10 mM $(NH_4)_2SO_4$.
**From ref. 14.

system in locust flight muscle during the rest → flight transition have shown that after 10 s of flight the pyruvate level increases from 0.064 to 0.36 μmol/g wet weight of tissue and the oxaloacetate level from 0.002 to 0.0034 μmol/g wet weight [16]. No significant change occurs in the levels of acetyl-CoA or ATP. If rapid equilibration of the mitochondrial and cytosolic pools is assumed [17] and the value for intracellular water volume in this tissue is taken as 420 μl/g wet weight [16], the concentration of pyruvate at rest and after 10 s flight is calculated as 0.15 and 0.86 mM, respectively. Alternatively if the rate of entry of pyruvate into locust flight muscle mitochondria is slow [18], this calculation over-estimates mitochondrial pyruvate concentration, especially after 10 s flight since the increased concentration of pyruvate probably arises from a transient imbalance between glycolytic and tricarboxylic acid fluxes. The range of the increase in concentration clearly coincides well with the apparent K_m for pyruvate which is observed for the flight muscle enzyme (0.25 mM, Table I) indicating that availability of pyruvate is likely to regulate the rate of oxaloacetate synthesis under these conditions.

In the case of acetyl-CoA, an upper limit for the mitochondrial concentration can be calculated as 0.42 mM if it is assumed that in flight muscle this metabolite is localised in the mitochondrion and that the mitochondrial water space is taken as 150 μl/g wet weight [16]. Although the value obtained appears far in excess of the apparent K_A observed for this activator (23 μM, Table II), this procedure is almost certain to overestimate the free mitochondrial acetyl-CoA concentration due to binding of the metabolite by proteins in this organelle. Furthermore, the apparent K_A may be increased if mitochondrial pH or pyruvate concentration are lower than suggested here (Fig. 1). The failure to observe a change in acetyl-CoA content on the rest

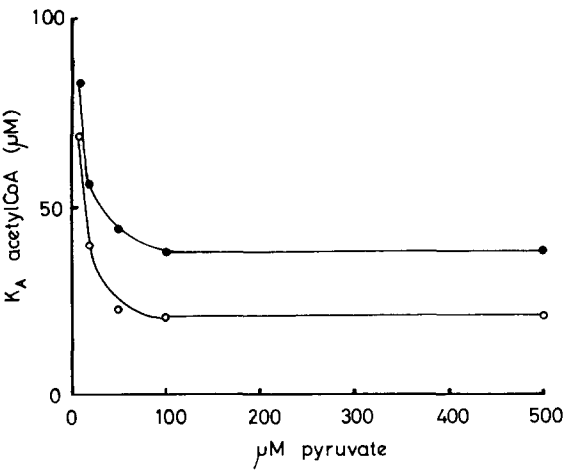

Fig. 1. Variation of the apparent K_A for acetyl-CoA with pyruvate concentration for pyruvate carboxylase from locust flight muscle at pH 6.9 (●—●) and pH 7.4 (○—○). The assay system contained, in 1.0 ml, 100 mM PIPES (potassium salt) (pH 6.9 or 7.4), 2 mM ATP, 5 mM $MgCl_2$, 50 mM $KHCO_3$, 2 units malate dehydrogenase, 0.15 mM NADH, the concentration of pyruvate as indicated, and acetyl-CoA at concentrations appropriate to the apparent K_A. After equilibration to 25° C the reaction was initiated by addition of 0.02—0.05 units partially purified locust flight muscle pyruvate carboxylase and the initial rate obtained from the decrease in absorbance at 340 nm. V was estimated from a plot of $1/v$ vs. $1/[\text{acetyl-CoA}]^2$ and apparent K_A and n_H were then obtained by plotting the data according to the Hill equation.

→ flight transition suggests however that any change in the rate of oxaloacetate synthesis is not likely to be mediated by this activator unless such an effect occurs as a secondary consequence of a change in pH or pyruvate concentration.

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