Pyruvate carboxylation in the rat heart

Role of biotin-dependent enzymes

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Pyruvate carboxylation in the isolated perfused rat heart was studied under steady-state conditions. A biotin deficiency resulting in a 90% decrease in myocardial pyruvate carboxylase left the pyruvate carboxylation rate unchanged. Pyruvate carboxylation in heart muscle must therefore take place by means of an enzyme which does not contain biotin. The kinetic properties and mass-action ratio of the NADP-linked malic enzyme in heart muscle can be taken as circumstantial evidence in favour of the role of malic enzyme in pyruvate carboxylation in myocardium.

INTRODUCTION

The tricarboxylic acid cycle in most tissues, including the heart, is subject to control at two levels: regulation of the cycle flux according to the fuel requirements of the mitochondrial respiratory chain (for a review, see Williamson & Cooper, 1980) and control of the combined metabolite pool size of the cycle (Peuhkurinen, 1984).

The size of the tricarboxylic acid-cycle intermediate pool in the isolated perfused heart increases after the addition of acetate, ketone bodies or fatty acids (Garland *et al.*, 1963; Randle *et al.*, 1970), upon aerobic arrest (Hiltunen & Hassinen, 1977), after ischaemia (Peuhkurinen *et al.*, 1983) and in diabetes (Bowman, 1966). Control over the metabolite pool size in the myocardium is effected by the operation of anaplerotic (Kornberg, 1966) and 'cataplerotic' mechanisms (for references, see Peuhkurinen, 1984).

It has been established during the last 10 years that pyruvate can undergo carboxylation to C₄ intermediates of the tricarboxylic acid cycle in muscle tissue. Since activity of pyruvate carboxylase (EC 6.4.1.1) has been found in myocardium, it has been suggested that this enzyme may be responsible for transferring the carbon skeleton of pyruvate to the oxaloacetate/malate pool (Davis *et al.*, 1980; Böttger *et al.*, 1969).

We have previously measured the concentrations of the reactants of NADP-dependent malic enzyme (EC 1.1.1.40) in both cytosolic and mitochondrial matrix spaces in the glucose-perfused heart by non-aqueous tissue-fractionation techniques (Sundqvist *et al.*, 1987). The data obtained demonstrate that the mass-action ratio of this reaction falls on the side of pyruvate carboxylation in both subcellular compartments by almost one order of magnitude. Thus the question arises as to whether pyruvate carboxylase or NADP-dependent malic enzyme is responsible for pyruvate carboxylation under conditions *in situ*.

Pyruvate carboxylase is a biotin-dependent enzyme, and biotin deficiency results in decreased enzyme activity in both the liver and muscle. The present data on the rate of pyruvate carboxylation in the perfused hearts of biotin-deficient rats show that the activity of pyruvate carboxylase is not sufficient to account for the rate of pyruvate carboxylation in the heart. Consequently the

carboxylation of pyruvate most probably takes place via NADP-dependent malic enzyme.

MATERIALS AND METHODS

Reagents

The enzymes were from Sigma, St. Louis, MO, U.S.A., and Boehringer, Mannheim, Germany. The chemicals were obtained from E. Merck, Darmstadt, Germany, and the nucleotides and coenzymes from Boehringer. Sodium [1-¹⁴C]pyruvate was purchased from Amersham International, Amersham, Bucks., U.K. Levamin 70 and Lipofundin S 20% infusion solutions were generously given by Leiras Pharmaceuticals, Turku, Finland. Inositol, cyanocobalamin, folic acid, choline chloride, biotin and egg-white powder were from Sigma, St. Louis, MO, U.S.A. Desivitol vitamin supplement was obtained from Orion Pharmaceutical Co., Helsinki, Finland. Ferropolar iron supplement was obtained from Medipolar Pharmaceuticals, Oulu, Finland.

Animals and diets

Female Sprague-Dawley rats from the Department's own stocks were used. After weaning, the rats were weight-paired into two groups and housed individually. During the experimental period of 21 days both groups were maintained on a liquid diet, the basic formula for which has been described previously (Lieber *et al.*, 1965; Lieber & DeCarli, 1970). Water was provided *ad libitum*. The energy content of the diet was 4.2 kJ/g of liquid diet, 60% being supplied by sucrose and 20% each by protein and fats. Lipofundin S 20% was used as the source of fats, consisting of 200 g of fractionated soya-bean oil, 15 g of soya-bean phosphatides and 25 g of glycerol per 1000 ml. The experimental group was supplied with amino acids solely in the form of dried egg-white protein, but the diet of the control group was supplemented with Levamin 70, containing 2.8 g of L-isoleucine, 4.4 g of L-leucine, 3.2 g of L-lysine, 4.4 g of L-phenylalanine, 2.0 g of L-threonine, 1.0 g of L-tryptophan, 3.2 g of L-valine, 2.2 g of L-histidine, 6.0 g of L-arginine, 16.0 g of L-alanine, 3.0 g of L-proline and 16.0 g of aminoacetic

acid per 1000 ml. Salts, vitamins, choline and folic acid were added in the amounts used by Lieber *et al.* (1965).

Perfusion methods and heart extracts

The rats were anaesthetized with sodium pentobarbital (80–100 mg/kg body wt., intraperitoneally) and injected intravenously with 500 i.u. of heparin 1 min before excision of the heart. The isolated hearts were perfused by a modification of the Langendorff procedure with Krebs–Henseleit (1932) bicarbonate solution, pH 7.4, in equilibrium with O₂/CO₂ (19:1). Perfusion with Krebs–Henseleit solution containing 5 mM-glucose, 12 i.u. of insulin/l and 0.2 mM-sodium pyruvate was initiated as an open system and continued for 10 min, after which the sodium [1-¹⁴C]pyruvate label was added and the perfusion continued for 10 min. At the end of the perfusion the heart was quick-frozen, extracted with HClO₄ and neutralized as described previously (Wollenberger *et al.*, 1960; Nuutinen *et al.*, 1981).

O₂ consumption and tricarboxylic acid-cycle flux

Perfusate O₂ concentrations were measured with a Clark-type electrode. The O₂ consumption was calculated by multiplying the arterio-venous O₂ concentration difference by the coronary flow. The tricarboxylic acid-cycle flux was estimated from the O₂ consumption, and was converted into acetyl-unit flux by dividing by 3.0 (Hiltunen & Hassinen, 1976; Latipää *et al.*, 1985).

Metabolite and specific-radioactivity determinations

The metabolites were determined enzymically by measuring the appearance or disappearance of NADH in an Aminco DW-2 dual-wavelength spectrophotometer, by using an $\epsilon_{340} - \epsilon_{385}$ value of 5.33×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. Citrate was measured with citrate lyase (EC 4.1.3.6) (Gruber & Möllering, 1966), malate essentially by the method of Williamson & Corkey (1969), glutamate with glutamate dehydrogenase (EC 1.4.1.4) (Bernt & Bergmeyer, 1970), 2-oxoglutarate as described by Narins & Passoneau (1970), and alanine as described by Grassl (1970). CoA and acetyl-CoA were measured with 2-oxoglutarate dehydrogenase and phosphotransacetylase (Tubbs & Garland, 1969). Aspartate and asparagine were measured with aspartate aminotransferase (EC 2.6.1.1), malate dehydrogenase (EC 1.1.1.37) and asparaginase (EC 3.5.1.1) (Bergmeyer *et al.*, 1970). ADP was determined as described by Adam (1963), and ATP by using hexokinase and glucose-6-phosphate dehydrogenase (Lamprecht & Trautschold, 1970).

After addition of 2 μ mol of the metabolites (except alanine) as a carrier, the radioactive metabolites were isolated from the HClO₄ extracts by ion-exchange chromatography on a Dowex-1 (formate form) column (LaNoue *et al.*, 1970) and eluted with an exponential gradient of formic acid and subsequently with ammonium formate. The radioactivity in the fractions was determined by liquid-scintillation counting. Determination of the malate concentration in the sample allowed the specific radioactivity of malate to be assessed. Alanine radioactivity was used as indicator of the specific radioactivity of intracellular pyruvate (Peuhkurinen & Hassinen, 1982; Peuhkurinen *et al.*, 1982). Alanine was not retarded in the anion-exchange resin under the conditions used, and therefore the alanine eluted in the void volume together with glucose was enzymically converted into pyruvate in the presence of an excess of 2-oxoglutarate and alanine aminotransferase (EC 2.6.1.2). The radioactivity of the pyruvate thus formed was analysed by re-chromatography on the Dowex-1 (formate form) column with added pyruvate as the carrier (Nuutinen *et al.*, 1981).

Pyruvate carboxylase and malic enzyme activity

Pyruvate carboxylase activity was determined as described by Böttger *et al.* (1969), and malic enzyme activity was measured in a reaction mixture containing 0.2 M-triethanolamine, pH 7.4, 0.5 mM-NADP⁺, 2 mM-MgCl₂ and tissue extract. The reaction was initiated by adding malate to a final concentration of 20 mM (Lin & Davis, 1974).

RESULTS AND DISCUSSION

Pyruvate carboxylation to C₄ intermediates of the tricarboxylic acid cycle is possible via two distinct routes: carboxylation to oxaloacetate catalysed by pyruvate carboxylase, or carboxylation to malate catalysed by malic enzyme. The reaction catalysed by mitochondrial malic enzyme is considered kinetically unfavourable (Swierczynski *et al.*, 1980; Lin & Davis, 1974), thus leaving pyruvate carboxylase as the major anaplerotic enzyme in this context (Davis *et al.*, 1980). Pyruvate carboxylase activity can be decreased by biotin deficiency (for a review, see Achuta Murthy & Mistry, 1972). Feeding rats on an avidin-rich diet for 3 weeks induced biotin deficiency, as seen by the decrease in pyruvate carboxylase activity (Table 1), which amounted to a total of 89% and 87% in the heart and liver tissue respectively and correlated well with earlier findings (Arinze & Mistry, 1971; Achuta Murthy & Mistry, 1972).

Table 1. Pyruvate carboxylase activity and oxidative-decarboxylation activity of NADP-dependent malic enzyme

The enzyme activities are given as μ mol/min per g fresh wt., and are means $\pm$ S.E.M. for four or five separate experiments at 30° C. Enzyme activities were measured as described in the Materials and methods section. NADP-dependent malic enzyme activity indicates total tissue-average activity. * $P < 0.01$, ** $P < 0.005$, for biotin-deficient group versus control group.

Conditions	Tissue	Pyruvate carboxylase activity	NADP-dependent malic enzyme activity
Control	Heart	0.116 $\pm$ 0.022	0.695 $\pm$ 0.051
	Liver	6.48 $\pm$ 1.24	1.67 $\pm$ 0.30
Biotin-deficient	Heart	0.0127 $\pm$ 0.0033**	0.764 $\pm$ 0.051
	Liver	0.816 $\pm$ 0.141*	2.74 $\pm$ 0.075

Table 2. O₂ consumption, tricarboxylic acid-cycle flux and pyruvate carboxylation rate

O₂ consumption was measured from the arteriovenous O₂-concentration difference and the coronary flow. The pyruvate carboxylation rate was calculated from the O₂ consumption and specific radioactivities of malate and intracellular pyruvate, as described in the Materials and methods section. Results are given as $\mu\text{mol}/\text{min per g dry wt.}$, and are means $\pm$ S.E.M. for five separate experiments.

Conditions	O ₂ consumption	Tricarboxylic acid-cycle flux	Pyruvate carboxylation rate
Biotin-deficient	23.7 $\pm$ 2.32	7.90 $\pm$ 0.773	0.387 $\pm$ 0.0828
Control	20.3 $\pm$ 0.80	6.77 $\pm$ 0.378	0.378 $\pm$ 0.0920

Table 3. Metabolite concentrations in isolated perfused hearts

Metabolites were determined in HClO₄ extracts of perfused freeze-clamped hearts by enzymic methods as described in the Materials and methods section. Values ($\mu\text{mol}/\text{g dry wt.}$) are given as means $\pm$ S.E.M. for five separate experiments: * $P < 0.05$, ** $P < 0.005$ compared with controls.

Metabolite	Control group	Biotin-deficient group
Acetyl-CoA	0.019 $\pm$ 0.003	0.015 $\pm$ 0.007
ADP	3.82 $\pm$ 0.15	4.17 $\pm$ 0.13
ATP	27.8 $\pm$ 1.7	26.8 $\pm$ 0.80
Glutamate	26.9 $\pm$ 1.5	27.3 $\pm$ 0.9
Aspartate	6.92 $\pm$ 0.51	9.14 $\pm$ 1.00
Asparagine	1.18 $\pm$ 0.14	1.49 $\pm$ 0.24
2-Oxoglutarate	0.67 $\pm$ 0.04	0.442 $\pm$ 0.033**
Citrate	0.945 $\pm$ 0.083	0.912 $\pm$ 0.11
Malate	1.02 $\pm$ 0.105	1.10 $\pm$ 0.11
Alanine	3.88 $\pm$ 0.212	4.44 $\pm$ 0.069*

Since C-1 and C-4 of malate and oxaloacetate are lost as CO₂ during the first turn in the tricarboxylic acid cycle, the rate of pyruvate carboxylation in the heart muscle can be estimated by a very straightforward calculation. Thus, in a steady state, ¹⁴C label efflux from the malate + oxaloacetate pool is equal to $F \cdot M$, where F is the tricarboxylic acid-cycle flux and M the specific radioactivity of malate. Although the total tricarboxylic acid-cycle pool is in a steady state, carboxylation of pyruvate can be considered unidirectional, in view of the kinetic properties of the enzyme and the mass-action ratio of malic enzyme in myocardium (Sundqvist *et al.*, 1987). Thus, in an isotopic and metabolic steady state, the pyruvate carboxylation rate (C) can be calculated as $C = F \cdot M / P$, where P is the specific radioactivity of intracellular pyruvate as estimated from the specific radioactivity of alanine.

The measured pyruvate carboxylation rate was in accordance with previous reports (Peuhkurinen *et al.*, 1982), but did not differ in the biotin-deficient group when measured under identical experimental conditions (Tables 1 and 2). There were no significant differences in the concentrations of known modulators of pyruvate carboxylase activity (acetyl-CoA, glutamate, ADP and ATP) (Davis *et al.*, 1980; McClure & Lardy, 1971; Scrutton & White, 1974) (Table 3) which could explain the sustained pyruvate carboxylation in the biotin-deficient group.

Malic enzyme activity was not affected by biotin deficiency (Table 1). The carboxylation rate of pyruvate was 0.39 and 0.38 $\mu\text{mol}/\text{min per g dry wt.}$ in the biotin-deficient and control myocardium respectively. Assuming a wet wt./dry wt. ratio of 7.2 and taking Q_7 (the temperature coefficient for 7 °C) = 1.6, the values given in Table 1 produce pyruvate carboxylase activities of 0.15 and 1.3 $\mu\text{mol}/\text{min per g dry wt.}$ in the biotin-deficient and control heart respectively at 37 °C. Thus the activity in the biotin-deficient group is insufficient to sustain the measured carboxylation rate.

If the rate of pyruvate carboxylation catalysed by malic enzyme is taken to be 15% of the measured maximal malate decarboxylation rate (Swierczynski *et al.*, 1980), if pyruvate carboxylation is assumed to occur in the mitochondria, and if the fraction of intramitochondrial enzyme activity is taken as 77% (Nolte *et al.*, 1972), the carboxylation rate catalysed by malic enzyme (estimated $Q_7 = 1.6$) can be calculated as 1.0 and 0.93 $\mu\text{mol}/\text{min per g dry wt.}$ for the biotin-deficient and control group respectively.

We have shown previously that under these experimental conditions the mass-action ratios of both intramitochondrial and extramitochondrial NADP-linked malic enzymes fall on the side of pyruvate carboxylation by more than one order of magnitude (Sundqvist *et al.*, 1987). One should note that, although the mass-action ratio of the reactants of the NAD⁺-linked malic enzyme (EC 1.1.1.38) is markedly different, this enzyme does not exist in the rat heart and need not be considered here (Morediath & Lehninger, 1984).

In the light of these data, it is evident that under the existing experimental conditions pyruvate carboxylase activity during biotin deficiency is decreased below the measured carboxylation rate in the intact myocardium. Simultaneously the mass-action ratio and the catalytic activity of malic enzyme are in favour of the carboxylation of pyruvate to malate, suggesting that this enzyme is anaplerotic rather than catabolic under most circumstances in the heart muscle.

Thus the fact that the amount (maximum activity) of pyruvate carboxylase is high enough to maintain the measured carboxylation rate in intact tissue can no longer be used as an argument for a significant metabolic role of pyruvate carboxylase in the heart muscle. Although it may be that the relative contributions of pyruvate carboxylase and malic enzyme are different in normal and biotin-deficient myocardium, the constancy of the carboxylation rate still indicates that the role of pyruvate carboxylase is small, evidently because the actual activity determined from the reactant and effector concentrations is low.

This investigation was supported by a grant from the Medical Research Council of the Academy of Finland. We thank Mrs. Kaisu Korhonen for her skilful technical assistance and Leiras Pharmaceuticals, Turku, Finland, for the generous gift of infusion solutions for preparation of the liquid diets.

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Received 21 September 1988/21 November 1988; accepted 25 November 1988