Role of NAD⁺-linked malic enzymes as regulators of the pool size of tricarboxylic acid-cycle intermediates in the perfused rat heart

Kaj E. SUNDQVIST,* Juhani HEIKKILÄ,† Ilmo E. HASSINEN* and J. Kalervo HILTUNEN*†

*Department of Medical Biochemistry and †Department of Clinical Chemistry, University of Oulu, SF-90220 Oulu, Finland

Cytosolic and mitochondrial concentrations of malate, 2-oxoglutarate, isocitrate and pyruvate in the isolated perfused rat heart were measured by non-aqueous tissue fractionation, taking the NADP-linked isocitrate dehydrogenases as indicator reactions for the free [NADPH]/[NADP⁺] ratios. The mass-action ratios of NADP-linked malic enzymes (EC 1.1.1.40) were found to be on the side of pyruvate carboxylation by more than one order of magnitude in both the cytosolic and the mitochondrial spaces in hearts perfused with glucose, whereas during propionate perfusion this ratio approached the equilibrium constant (K_{eq}) of malic enzyme. The results consequently indicate that the NADP-linked malic enzymes cannot be responsible for the feed-out (cataplerotic) reactions from the tricarboxylic acid cycle which occur during glucose perfusion. Only when other anaplerotic fluxes into the cycle are high, as during propionate oxidation, which results in accumulation of tricarboxylic acid-cycle intermediates, is a steady state reached which allows efflux via the malic enzyme.

INTRODUCTION

The combined pool size of tricarboxylic acid-cycle intermediates in heart muscle can undergo a rapid change if the fuel substrates or the work load of the heart are altered (Garland *et al.*, 1963; Randle *et al.*, 1970; Safer & Williamson, 1973; Davis & Bremer, 1973; Hiltunen & Hassinen, 1977). This demonstrates the existence of reactions feeding carbon skeletons into and out of the tricarboxylic acid cycle. These opposing phenomena are known as anaplerosis and cataplerosis respectively (Kornberg, 1966; Peuhkurinen, 1984). The anaplerotic mechanisms comprise pyruvate or propionate carboxylations, coupled transamination reactions of aspartate and alanine aminotransferases, glutamate dehydrogenase and the purine nucleotide cycle, all of which have been shown to occur in rat heart muscle (Davis & Quastel, 1964; Davis & Bremer, 1973; Safer & Williamson, 1973; Takala *et al.*, 1980; Nuutinen *et al.*, 1981). The possible cataplerotic reactions include those of intramitochondrial NAD(P)- (EC 1.1.1.39) or NADP-linked malic enzymes, cytosolic NADP-linked malic enzyme, oxaloacetate decarboxylase, glutamate dehydrogenase or coupled transamination reactions. Of these enzyme systems, oxaloacetate decarboxylase (Wojtczak & Walajtys, 1974) and NAD(P)-linked malic enzyme (Nagel *et al.*, 1980) seem to be absent from rat heart muscle. The relative significances of these anaplerotic and cataplerotic mechanisms for the regulation of the pool size of tricarboxylic acid-cycle intermediates and energy metabolism in the myocardium are nevertheless unclear at present.

The present work uses tissue fractionation in non-aqueous media to examine anaplerosis and cataplerosis in the intact perfused rat heart (Elbers *et al.*, 1974; Kauppinen *et al.*, 1980). This method allows metabolite concentrations in both the cytosolic and the mitochondrial spaces to be calculated. The estimated free [NADPH]/[NADP⁺] ratios and concentrations of reactants of NADP-linked malic enzymes reveal that the mass-action ratios are on the side of pyruvate carboxyl-

ation by more than one order of magnitude in the glucose-perfused heart, but approached near-equilibrium conditions during propionate perfusion.

MATERIALS AND METHODS

Reagents

The enzymes, nucleotides and coenzymes were purchased from Boehringer G.m.b.H, Mannheim, Germany, and Sigma Chemical Co., St. Louis, MO, U.S.A. Propionic acid was obtained from Fluka A.G., Buchs, Switzerland. The routine chemicals were obtained from E. Merck A.G., Darmstadt, Germany.

Animals and perfusion methods

Female Sprague–Dawley rats (weighing 180–230 g) from the Department's own stock were used. After anaesthesia with an intraperitoneal injection of pentobarbital (60 mg/kg), heparin was injected intravenously (500 mg/kg) and the heart was excised. The isolated hearts were perfused by the Langendorff (1895) procedure with Krebs–Henseleit (1932) solution in equilibrium with O₂/CO₂ (19:1). The perfusion medium contained 2.5 mM-Ca²⁺, 5 mM-glucose, 12 units of insulin/l and 2 mM-propionate as indicated. The hydrostatic perfusion pressure was 7.85 kPa (80 cmH₂O).

Tissue-fractionation procedures and metabolite determinations

The hearts were quick-frozen with aluminium clamps cooled with liquid N₂ (Wollenberger *et al.*, 1960) and freeze-dried at –55 °C. The tissue was then fractionated in a non-aqueous medium (Elbers *et al.*, 1974; Kauppinen *et al.*, 1980). The marker enzymes for the subcellular compartments, citrate synthase for the mitochondria and phosphoglycerate kinase for the cytosolic spaces, were measured (Shepherd & Garland, 1969; Bücher, 1970). Protein concentrations were determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

A portion of each fraction collected from a CCl_4 /heptane gradient was evaporated to dryness, and 0.25 ml of 0.05 M-HCl was added together with a small amount of glass beads. After mixing, the samples were kept at 100 °C for 3 min, cooled and partially neutralized with 50 μl of 0.2 M-KOH. The metabolites in these extracts were allowed to react stoichiometrically with nicotinamide nucleotides (Lowry & Passonneau, 1972), followed by amplification with enzymic cyclings (Lowry *et al.*, 1961; Kato *et al.*, 1973).

Calculations

The frequency of a given metabolite in fractions from a density gradient is defined by eqn. (1):

$$Y_j = X_1 \cdot C_{1j} + X_2 \cdot C_{2j} + \dots + X_n \cdot C_{nj} + e_j \quad (1)$$

where Y_j is the frequency sum of the metabolite in the j th fraction, $C_{1j}, C_{2j}, \dots, C_{nj}$ are the frequencies of the marker enzymes of subcellular compartments 1, 2, ..., n , $X_1, X_2, \dots, X_n$ are the fractions of the metabolite in the same compartments 1, 2, ..., n , and e_j is an error term.

$X_1, X_2, \dots, X_n$ were solved by fitting eqn. (1) to the set of data points by an iterative Gauss-Newton algorithm (Magar, 1972; Bronfman *et al.*, 1982) on a minicomputer (Data General Nova 3). Constraints ($0 \leq X \leq 1$) were introduced into the program to prevent negative values of X .

Once the values of X , the total concentration in the tissue and the volumes of the cytosolic and mitochondrial water spaces are known, it is possible to calculate the cytosolic and mitochondrial metabolite concentrations. The cytosolic and mitochondrial water volumes were taken as 4.43 and 1.01 $\mu\text{l}/\text{ml}$ of protein respectively (Kauppinen, 1983). The statistical significances of the data were calculated by Student's t test.

RESULTS

Metabolite gradients

The mitochondrial concentrations of all the metabolites measured here were found to be higher than in the cytosol in both the glucose- and the propionate-perfused hearts. In the former, pyruvate had the lowest concentra-

tion gradient across the mitochondrial membrane and isocitrate the highest (Table 1). Infusion of 2 mM-propionate resulted in 53- and 30-fold increases in malate concentrations in the cytosol and mitochondria respectively, whereas the cytosolic 2-oxoglutarate concentration remained unchanged and its mitochondrial concentration decreased by 45%. The present data on 2-oxoglutarate compartmentation in the glucose-perfused heart are in line with a previous report (Kauppinen *et al.*, 1982). The pyruvate concentrations measured here are high compared with the tissue-average concentration reported by Kauppinen *et al.* (1982) and Peuhkurinen *et al.* (1982). The difference could be due to infusion of insulin in the present experiments.

Redox state of NADP(H) and mass-action ratios of malic enzymes

The activities of the NADP-linked isocitrate dehydrogenases are high in the myocardium, 86% of this activity in the rat heart being due to the mitochondrial isoenzyme (Pette, 1966). Goebell & Klingenberg (1964) propose that the NAD-linked isocitrate dehydrogenase may catalyse a non-equilibrium reaction of the tricarboxylic acid cycle under the conditions prevailing in mitochondria, whereas the reaction catalysed by the NADP-linked isocitrate dehydrogenase is near equilibrium. This notion is supported by a rapid labelling of C-6 of citrate (and isocitrate) via reversal of the NADP-linked isocitrate dehydrogenase during perfusion of isolated hearts with [^{14}C]bicarbonate (Randle *et al.*, 1970; Nuutinen *et al.*, 1981; Peuhkurinen & Hassinen, 1982). Table 2 gives the free $[\text{NADPH}]/[\text{NADP}^+]$ ratios as calculated from the 2-oxoglutarate and isocitrate concentrations and the equilibrium constant for isocitrate dehydrogenase, which is independent of pH (Krebs & Veech, 1969).

Usefulness of the NADP-linked isocitrate dehydrogenase as a redox indicator can be evaluated by a comparison of the redox-potential values for the mitochondrial NADPH/NADP⁺ couple calculated from the isocitrate dehydrogenase system on one hand and from the energy-linked NAD(P) transhydrogenase on the other. At a matrix pH of 7.6 and under comparable

Table 1. Compartmentation of pyruvate, 2-oxoglutarate, malate and isocitrate in the isolated perfused rat heart

The cytosolic and mitochondrial components of freeze-clamped hearts were enriched by subcellular fractionation in a non-aqueous density gradient, and the contents of metabolites and activities of marker enzymes were measured. The compartmental concentrations of the metabolites were calculated by an iterative computer program designed to fit a compartment-distribution model to the data points, as described in the Materials and methods section. The values are concentrations (mM) given as means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Statistical significance is indicated by * $P < 0.10$ and ** $P < 0.005$ respectively (hearts perfused with glucose versus hearts perfused with propionate).

Metabolite	Perfusion . . .	Concn. (mM)			
		Glucose		Glucose + propionate	
		Cytosolic	Mitochondria	Cytosol	Mitochondria
Pyruvate		0.73 $\pm$ 0.09 ($n = 5$)	1.22 $\pm$ 0.40 ($n = 5$)	1.15 $\pm$ 0.23 ($n = 4$)*	2.55 $\pm$ 1.13 ($n = 4$)
Malate		0.20 $\pm$ 0.03 ($n = 5$)	0.87 $\pm$ 0.13 ($n = 5$)	10.7 $\pm$ 3.0 ($n = 5$)**	25.9 $\pm$ 5.1 ($n = 5$)**
2-Oxoglutarate		0.12 $\pm$ 0.04 ($n = 5$)	0.84 $\pm$ 0.21 ($n = 5$)	0.11 $\pm$ 0.03 ($n = 5$)	0.46 $\pm$ 0.06 ($n = 5$)*
Isocitrate		0.023 $\pm$ 0.011 ($n = 4$)	0.25 $\pm$ 0.04 ($n = 4$)	0.029 $\pm$ 0.012 ($n = 5$)	0.31 $\pm$ 0.08 ($n = 5$)

Table 2. Calculated free [NADPH]/[NADP⁺] ratios and mass-action ratios for malic enzyme in mitochondrial and cytosolic compartments of the isolated perfused rat heart

Free [NADPH]/[NADP⁺] ratios were calculated from the data of Table 1, the K_{eq} of isocitrate dehydrogenase being taken as 0.91 M (Krebs & Veech, 1969). The CO₂ concentration in the perfusion medium was 1.27 mM, and the intracellular CO₂ concentration was assumed to be the same. The mass-action ratios of malic enzyme were calculated as $\Gamma' = [\text{NADPH}] \cdot [\text{CO}_2] \cdot [\text{pyruvate}] / [\text{NADP}^+] \cdot [\text{malate}] = K_{eq, \text{ICDH}} \cdot [\text{isocitrate}] \cdot [\text{pyruvate}] / [2\text{-oxoglutarate}] \cdot [\text{malate}]$, where ICDH = isocitrate dehydrogenase.

	Perfusion . . .		Glucose + propionate	
	Cytosolic	Mitochondrial	Cytosolic	Mitochondrial
Free [NADPH]/[NADP ⁺] (molar ratio)	137	213	188	779
10 ⁻² × Mass-action ratio of malic enzyme (mM)	630	380	25.6	60.4

conditions (Kauppinen, 1983), the mitochondrial free [NADPH]/[NADP⁺] ratio given in Table 2 is equivalent to a redox potential (E_h) of -433 mV, calculated from an $E'_{m7.0}$ value of -343 mV (Sies, 1982), corrected to pH 7.6. In addition to the trans-membrane ΔpH , the mitochondrial membrane potential has also been determined under comparable conditions, and these give an electrochemical potential for the protons ($\Delta\mu\text{H}^+$) of 15.5 kJ/mol, equivalent to a protonmotive force (Δp) of -161 mV (Kauppinen *et al.*, 1983; Kauppinen, 1983). The energy-linked (proton-pumping) NAD(P) transhydrogenase is driven by this Δp (Rydström, 1977). The E_h of the mitochondrial free NADH/NAD⁺ couple is -303 mV under the same conditions (Kauppinen *et al.*, 1983), and the E_h of the free NADPH/NADP⁺ couple becomes equal to -303 mV + (-161 mV) = -464 mV. This means that there is a difference of one order of magnitude between the experimentally determined free [NADPH]/[NADP⁺] ratio of 213 and the ratio of 2050 representing the equilibrium state of the transhydrogenase. The transhydrogenase equilibrium would then result in an [NADPH]/[NADP⁺] ratio so high that it cannot be reached, owing to kinetic limitation of the reaction. Because ¹⁴CO₂-incorporation data from several laboratories indicate that the isocitrate dehydrogenase reactions as a whole are in equilibrium (Randle *et al.*, 1970), although the NAD⁺-linked is not (Hansford & Johnson, 1975), the NADP⁺-linked isocitrate dehydrogenase is used as an indicator enzyme of the free [NADPH]/[NADP⁺] ratio in the present study.

The estimated cytosolic and mitochondrial mass-action ratios for NADP-linked malic enzyme were roughly one order of magnitude above the K_{eq} in the hearts perfused with glucose ($K_{eq} = 3.44 \times 10^{-2}$ M; $t = 38^\circ\text{C}$; $I = 0.25$; K_{eq} is independent of pH) (Krebs & Veech, 1969), but upon addition of propionate the mass-action ratio decreased in both cell compartments and the calculated values approached K_{eq} (Table 2). It has been shown that there is cytosolic sub-compartmentation of pyruvate in the myocardium (Peuhkurinen *et al.*, 1983), although the proportional distribution into these is not known. In the present context the relatively large deviations make the interpretation of the results straightforward without consideration of cytosolic sub-compartmentation.

DISCUSSION

The intermediates of the tricarboxylic acid cycle can be regarded as catalysts during the oxidation of acetyl-CoA to CO₂, in which the intermediates are metabolized to each other without any change in the total pool size. Regulation of the pool size by means of anaplerotic and cataplerotic reactions is involved not only in feed-back control of the turnover of the cycle itself (LaNoue *et al.*, 1972; Safer & Williamson, 1973; Bremer & Davis, 1974), but also in the transmission of signals between the mitochondria and the cytosol. The latter is illustrated by the citrate inhibition of phosphofructokinase during fatty acid oxidation, which has also been demonstrated in heart muscle (Garland *et al.*, 1963; Garland & Randle, 1964; Kauppinen *et al.*, 1982). Furthermore, the conversion of the carbon skeletons of many amino acids into alanine, their net oxidation and also the total oxidation of odd-numbered fatty acids in muscle tissue necessitate mechanisms which convert C₄ intermediates of the tricarboxylic acid cycle into C₃ compounds, i.e. cataplerotic reactions.

Pyruvate is carboxylated to malate/oxaloacetate both in isolated heart mitochondria (Davis *et al.*, 1980) and in the isolated perfused rat heart itself, in which the rate is approx. 1 μmol/min per g dry wt. (Nuutinen *et al.*, 1981; Peuhkurinen *et al.*, 1983). The fact that the mass-action ratios of both cytosolic and mitochondrial malic enzymes are on the side of the carboxylation of pyruvate, irrespective of whether the isocitrate dehydrogenases or the NAD(P) transhydrogenase is used to estimate the free [NADPH]/[NADP⁺] ratio, in hearts perfused with glucose suggests that incorporation of CO₂ into malate via these reactions is possible. Also, the magnitude of the deviation of the mass-action ratios from the K_{eq} values suggests that the actual flux through malic enzymes towards carboxylation must be operating near the maximum kinetically possible under these conditions. Total malic enzyme activity in rat heart muscle is high, about 1.8 unit/g wet wt., as measured *in vitro* towards decarboxylation (Brdiczka & Pette, 1971), but the rate towards carboxylation is much lower. It also requires unphysiologically high concentrations of pyruvate and bicarbonate in order to be significant, about 6% of the rate of malate decarboxylation being achieved with

50 mM-pyruvate and 200 mM-bicarbonate (Lin & Davis, 1974). It is worth noting in this context that the NAD(P)-linked malic enzyme which exists in the guinea-pig and rabbit myocardium (Lin & Davis, 1974) is effectively substrate-inhibited by NADH, making the reaction kinetically irreversible (Davisson & Schulz, 1985). In view of this, it remains an open question whether pyruvate carboxylation in the myocardium occurs via malic enzyme or pyruvate carboxylase.

Since a continuous feed-in of carbon skeletons into the tricarboxylic acid cycle occurs in hearts perfused with glucose without any major changes in total pool size (Takala *et al.*, 1980; Nuutinen *et al.*, 1981), feed-out reactions must be taking place at the same time, and this may be effected by phosphoenolpyruvate carboxykinase, although its activity in muscle tissue is low (Nolte *et al.*, 1972; Nagel *et al.*, 1980). Snell & Duff (1977) have found that the phosphoenolpyruvate carboxykinase inhibitor mercaptopicolinate inhibits alanine production from valine and isoleucine in isolated diaphragm muscle from starved rats, and it is also known to inhibit the incorporation of radioactivity from [¹⁴C]lactate into glycogen in frog sartorius muscle (Connett, 1979). However, mercaptopicolinate may have multiple sites of action in muscle tissue (Palmer *et al.*, 1984).

Tricarboxylic acid-cycle intermediates accumulate in the heart muscle during propionate oxidation (Davis & Quastel, 1966). Malate accounts for most of this, and Dynnik *et al.* (1984) suggest that this is a consequence of a forward inhibition of citrate synthase by malate. Lee & Davis (1979) did not find that mercaptopicolinate had any effect on the output of pyruvate, lactate or alanine in rat hindquarter preparations perfused with propionate. Furthermore, hydroxymalonate, an inhibitor of NADP-linked malic enzyme, completely inhibits the metabolism of propionate to pyruvate in isolated rat heart mitochondria with a concomitant accumulation of malate, which indicates that malic enzyme is involved in cataplerosis (Hiltunen & Davis, 1981). To summarize the earlier results and the present findings, it seems apparent that the low activity of phosphoenolpyruvate carboxykinase in the myocardium is not sufficient to cope with the rate of anaplerosis during propionate perfusion. Consequently, an accumulation of tricarboxylic acid-cycle intermediates must occur until a steady state is reached, under which cataplerosis via malic enzymes is possible.

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