Biochimica et Biophysica Acta, 616 (1980) 10—21

BBA 69132

PURIFICATION AND SOME PROPERTIES OF EXTRAMITOCHONDRIAL MALIC ENZYME FROM RAT SKELETAL MUSCLE

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(Received April 4th, 1980)

Key words: Malic enzyme; Pyruvate carboxylation; (Skeletal muscle)

Summary

Extramitochondrial malic enzyme (L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) has been isolated from postmitochondrial supernatant of rat skeletal muscle, by $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-cellulose, Sepharose 6B, ADP-Sepharose and Ultrogel AcA-34 to apparent homogeneity as judged from polyacrylamide gel electrophoresis. Specific activity of purified enzyme was 20 μmol · min^{-1} per mg protein, which corresponds to about 3000-fold purification.

The molecular weight of the native enzyme was determined by gel filtration to be 264 000. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis showed one polypeptide band of molecular weight 63 000. Thus, it appears that the native protein is a tetramer composed of identical molecular weight subunits.

The isoelectric point of the isolated enzyme was at pH 6.15.

The enzyme was shown to carboxylate pyruvate in the presence of high concentrations of bicarbonate and pyruvate at about 80% of the rate of the forward reaction.

The K_m values, determined at pH 7.2 for malate and NADP, were 0.125 mM and 11 μM, respectively. The K_m values for pyruvate, NADPH and bicarbonate were 4.0 mM, 6.6 μM and 24 mM, respectively.

The optimum pH for carboxylation reaction was at pH 7.1. The optimum pH for decarboxylation reaction varied with the malate concentration. The purified malic enzyme catalyzed the decarboxylation of oxaloacetate at pH 4.5.

In a system consisting of isolated rat skeletal muscle mitochondria, pyruvate, bicarbonate and NADPH, cytoplasmic malic enzyme is able to replace added

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

malate in stimulating oxidation of acetyl-CoA formed by oxidative decarboxylation of pyruvate.

It is suggested that extramitochondrial malic enzyme might be one of the enzymes involved in the anaplerotic supply of Krebs cycle intermediates in skeletal muscle.

Introduction

Lee and Davis [1] have shown recently that radioactivity from $NaH[^{14}C]O_3$ is incorporated into Krebs cycle intermediates by glucose-perfused rat hindquarters. The extent of ^{14}C incorporation increases markedly while the tissue pyruvate is elevated [1]. Thus, it is possible to conclude that carboxylation of pyruvate in skeletal muscle may provide a pathway for repletion of citric acid cycle intermediates. However, the mechanism for the observed incorporation of ^{14}C has not been definitely established. Early work by Spydevold et al. [2] suggested that pyruvate carboxylase (pyruvate:carbon dioxide ligase (ADP-forming), EC 6.4.1.1) can provide an increase in citrate cycle intermediates under conditions when the acetyl-CoA is elevated. It is generally believed that phospho*enol*pyruvate carboxykinase and malic enzyme are also capable of net feed-in of citric acid cycle intermediates. Although it is known that these enzymes are present in varying amounts in the muscle from various sources [3,4], their purification or characterization has not yet been reported. We have shown recently [5] that malic enzyme purified from rat skeletal muscle mitochondria was able to catalyze the reductive carboxylation of pyruvate. However, the kinetics of mitochondrial malic enzyme being very unfavorable for the reductive carboxylation of pyruvate [5], makes it unlikely that this enzyme could catalyze net carboxylation under physiological conditions. In this respect mitochondrial malic enzyme from rat skeletal muscle resembles mitochondrial malic enzyme from other sources [6—9]. On the other hand, Simpson and Estabrook [8] and Frenkel [7] reported that extramitochondrial malic enzyme from adrenal cortex and bovine brain might produce malate from pyruvate. As a substantial activity of extramitochondrial NADP-linked malic enzyme is present in skeletal muscle [3] it seems likely that this enzyme may be responsible for carboxylation of pyruvate to malate. To check this possibility it was necessary to isolate and characterize to extramitochondrial malic enzyme from skeletal muscle.

Materials and Methods

The following chemicals were purchased from Sigma Chemical Co.: NADP, NADPH, malic acid, pyruvic acid (sodium salt), *cis*-oxaloacetic acid, phenazine methosulphate, nitro blue tetrazolium, malate dehydrogenase, glutamate dehydrogenase. Sepharose 6B and ADP-Sepharose were from Pharmacia Fine Chemicals. Ultrogel AcA-34 and ampholine were from LKB, Sweden. DEAE-cellulose was from Whatman Biochemicals Ltd. Protein standards were from Serva-Feinbiochemica, Heidelberg. All other chemicals were of the highest purity available commercially.

Male Wistar rats maintained on a commercial complete diet were used for experiments.

Rat skeletal muscle obtained from the hind legs immediately after decapitation was freed of connective and adipose tissue, minced finely with scissors and rinsed throughly with isotonic KCl. Mitochondria were prepared as described previously [10]. Postmitochondrial supernatant was additionally centrifuged at 20 000 × *g* for 30 min and used as a source of extramitochondrial malic enzyme.

Protein concentrations were determined by the biuret method, or after extensive purification of the enzyme by the method of Spector [11].

Enzyme assay

The enzyme activity at all purification steps was followed spectrophotometrically with Specord UV Vis recording spectrophotometer by observing the appearance of NADPH at 340 nm and 30°C. The standard reaction mixture for malic enzyme assay (final volume 1 ml) contained: 50 mM Tris-HCl, pH 7.8, 1 mM $MnCl_2$, 0.5 mM NADP, 12 mM L-malate and the enzyme in amounts which caused the increase of absorbance at the range 0.1—0.2 per min. Oxaloacetate decarboxylase activity was measured essentially as described by Kosicki [12] by following the disappearance of oxaloacetate at 260 nm and 22°C. The reaction mixture contained: 1 mM oxaloacetate/0.2 M potassium acetate buffer (pH 4.5)/1 mM $MnCl_2$/malic enzyme, the final volume was 1 ml. The effect of NADP on oxaloacetate decarboxylase activity was assayed in conditions described by Hsu and Lardy [13] or by following the disappearance of oxaloacetate at 290 nm in conditions described above, except that oxaloacetate was at 4 mM concentration.

Determination of molecular weight by gel filtration was performed as described by Andrews [14] with a column (2.5 × 95 cm) of Sepharose 6B, equilibrated with 10 mM Tris-HCl (pH 7.8)/2 mM EDTA (pH 7.8).

Molecular weight determination by SDS-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn [15]. The samples were incubated at 100°C for 2 min in 0.01 M phosphate buffer (pH 7.1) containing: 1.5% SDS and 0.05% dithiothreitol and then dialysed against 0.01 M phosphate buffer (pH 7.1) containing: 0.1% SDS, 0.01% dithiothreitol and 30% glycerol at 30°C for 10 h. Protein (15—30 μg) in a vol. of 40 μl was applied on 7.5% polyacrylamide gel.

To perform electrophoretic analysis, native enzyme was dialyzed against 0.01 M Tris-glycine buffer (pH 8.5). The sample of the enzyme (30—40 μg) in a vol. of 40 μl was applied on 5% polyacrylamide gel prepared in buffer containing 0.1 M Tris-HCl (pH 8.5). Electrophoresis was carried out in glass tubes (0.5 × 10 cm) at 1 mA per gel for 90 min at 4°C. 0.1 M Tris-glycine (pH 8.5) was used as electrode buffer. Protein in the gels was stained with Coomassie blue. Malic enzyme activity in the gels after electrophoresis was determined by method of Henderson [16].

Isoelectric focusing was carried out in sucrose density gradient according to the LKB manual with a narrow range of ampholine pH 5—7, in LKB model 8100 apparatus. The enzyme (about 10 units) was dialyzed against 1% glycine before application to the column. Electrofocusing was performed at 600 V for

48 h at 4°C, and the pH of the 2.5-ml fractions was measured immediately after elution at 4°C.

Velocity sedimentation studies were carried out at 20°C at a speed of 60 000 rev./min on a MSE Centriscan 75 analytical ultracentrifuge equipped with schlieren optics (knife edge angle 75°) according to Schachman [17]. Sedimentation values were obtained at a protein concentration of 2 mg/ml in 10 mM Tris-HCl/2 mM EDTA (pH 8.2).

Purification of extramitochondrial malic enzyme

$(NH_4)_2SO_4$ fractionation. All operations in this and subsequent steps were carried out in the cold (2—5°C). The pH of postmitochondrial supernatant was adjusted to 7.8 by adding concentrated Tris solution. The solution was then adjusted to 50% saturation of $(NH_4)_2SO_4$ by gradual addition of a corresponding amount (313 g per l) of solid $(NH_4)_2SO_4$. After stirring for 45 min the suspension was centrifuged at 2500 × *g* for 60 min. The resulting clear supernatant was brought to 70% saturation of $(NH_4)_2SO_4$ by the gradual addition of 137 g per l of solid $(NH_4)_2SO_4$. After stirring for 45 min the suspension was centrifuged at 2500 × *g* for 60 min. The precipitate was dissolved in a minimum volume of 10 mM Tris-HCl (pH 7.8) containing 2 mM EDTA and dialyzed against 200 vols. of the same buffer during 24 h with one change of buffer. Insoluble material was removed by centrifugation at 15 000 × *g* for 20 min. The material so obtained was used for determination of enzyme activity and protein concentration, as the enzyme activity was inhibited by $(NH_4)_2SO_4$ similar to the malic enzyme from pig heart [18]. While adding $(NH_4)_2SO_4$ the pH was kept constant at 7.8 by appropriate additions of 5 mM NH_4OH.

DEAE-cellulose chromatography. The DEAE-cellulose (type DE-32) was washed with 20 vol. of 1 M Tris-base and then repeatedly with deionized water and equilibrated by washing with a solution containing 10 mM Tris-HCl/2 mM EDTA (pH 7.8). The dialyzed enzyme solution was applied to a column (2 × 40 cm) packed with DEAE-cellulose. After the protein solution had entered the column, washing was started with the solution containing 10 mM Tris-HCl (pH 7.8) /2 mM EDTA. The washing procedure was continued until a large protein peak devoid of malic enzyme activity, but containing the bulk of malate dehydrogenase and lactate dehydrogenase activities emerged from the column. Malic enzyme activity was eluted by a linear gradient of KCl concentration with a solution containing 10 mM Tris-HCl (pH 7.8)/2 mM EDTA (pH 7.8) and 0.4 M KCl buffered with 10 mM Tris-HCl (pH 7.8)/2 mM EDTA (pH 7.8). 250 ml of the above solutions were sufficient to complete the elution. Fractions (4 ml) were collected from the column at a rate 20 ml/h. Malic enzyme activity was eluted as a single symmetrical peak included in a broad protein fraction. The active fractions were pooled and concentrated with a collodion bag apparatus (Schleicher and Schuell).

Sepharose 6B chromatography. Concentrated eluate from DEAE-cellulose was applied to a column of Sepharose 6B (2.5 × 95 cm) equilibrated with 10 mM Tris-HCl/2 mM EDTA (pH 7.8). Elution was performed with the solution containing 10 mM Tris-HCl/2 mM EDTA (pH 7.8) at a rate of 10 ml/h. 4 ml fractions were collected. The washing procedure was continued until a protein peak emerged from the column. Malic enzyme activity was eluted as a single

symmetrical peak. Active fractions from this column were pooled and concentrated as described above.

ADP-Sepharose chromatography. Concentrated material from the Sepharose 6B column was applied on the ADP-Sepharose column (1 × 10 cm) equilibrated with 10 mM Tris-HCl/2 mM EDTA (pH 7.8). The column was washed with 10 mM Tris-HCl/2 mM EDTA (pH 7.8) until a protein peak devoid of malic enzyme activity was eluted. Malic enzyme activity was eluted with 0.3 mM NADP. Fractions (2.5 ml) were collected from the column at a rate 10 ml/h. The active fractions were pooled and concentrated as described above. Polyacrylamide gel electrophoresis of the enzyme at this stage of purification in the presence of SDS showed one major and a few faint bands. Therefore, the enzyme was further purified by gel filtration using Ultrogel AcA-34.

Ultrogel AcA-34 chromatography. The enzyme solution from the previous step was applied to a column of Ultrogel AcA-34 (2 × 90 cm) equilibrated previously with 10 mM Tris-HCl/2 mM EDTA (pH 7.8) and eluted with the same buffer at a rate of 5 ml/h. 3-ml fractions were collected. Active fractions were pooled and concentrated as described above.

Results

Table I summarizes the purification of extramitochondrial malic enzyme from rat skeletal muscle. At a yield of 13% the enzyme was purified about 3000-fold. The specific activity measured in the decarboxylation direction was about 20 $\mu mol \cdot min^{-1}$ per mg protein. It is higher than the specific activity reported by Bartholome et al. [18] for partially purified malic enzyme from pig heart and lower than specific activity reported by Hsu and Lardy [13] and Wada et al. [19] for crystalline extramitochondrial malic enzyme from pigeon and rat liver, respectively.

TABLE I

PURIFICATION OF EXTRAMITOCHONDRIAL MALIC ENZYME FROM RAT SKELETAL MUSCLE

Enzyme was isolated from 300 g skeletal muscle. For other conditions see text.

Step	Volume (ml)	Total protein (mg)	Specific activity (nmol · min^{-1} · mg^{-1})	Total activity ($\mu mol \cdot min^{-1}$)	Yield (%)	Purification (-fold)
Cytosol	680	9520	6.3	60.3	100	1
$(NH_4)_2SO_4$(50—70%) fractionation	87	2784	17.0	47.4	78.5	2.7
DEAE-cellulose chromatography	45	207.4	175.0	36.3	60.1	27.7
Sepharose 6B chromatography	38	37.4	680.0	25.4	42.2	107.9
ADP-Sepharose chromatography	12	1.9	8060.0	15.3	25.4	1279.4
Ultrogel AcA-34 chromatography	22	0.4	20 000.0	8.0	13.3	3174

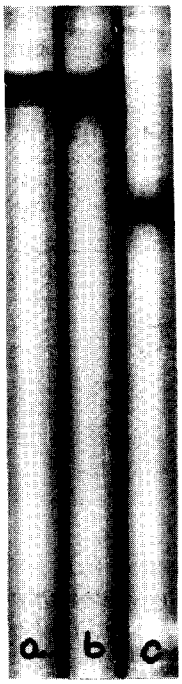

Fig. 1. Polyacrylamide gel electrophoresis of native (a and b) and SDS-treated extramitochondrial malic enzyme (c). Experimental conditions as described under Materials and Methods. a, stained for enzyme activity with tetrazolium blue; b and c stained for protein with Coomassie blue.

Homogeneity and molecular weight. Homogeneity of purified malic enzyme was demonstrated by polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide gels produced one protein band and a corresponding band appeared in an identical gel stained for enzyme activity (Fig. 1a and b). When electrophoresis was performed in the presence of SDS one protein band was obtained (Fig. 1c).

Analytical ultracentrifugation of the purified enzyme revealed a single, symmetrical peak which remained constant over a period of 60 min running time, indicating a high degree of protein homogeneity. The sedimentation coefficient was calculated to be $s_{20,w} = 9.7$. It is similar to that reported by Hsu and Lardy [13] for crystalline extramitochondrial malic enzyme from pigeon liver.

The apparent molecular weight of the extramitochondrial malic enzyme from rat skeletal muscle estimated by gel filtration through a calibrated Sepharose 6B column was 264 000 (range 250 000—280 000 in four experiments). The molecular weight of SDS-treated malic enzyme was estimated to be 63 000 (range 61 700—64 600 in three experiments). These results demonstrate that the enzyme, similarly to enzyme isolated from pigeon liver [20] is a tetramer consisting of subunits of equal molecular weight.

Isoelectric point. The isoelectric point of rat skeletal muscle extramitochondrial malic enzyme was 6.15 as estimated by isoelectrofocusing method. A similar value was reported by Wada et al. [19] for rat liver extramitochondrial malic enzyme. However, according to Batholome et al. [18] the isoelectric

TABLE II

REVERSIBILITY OF THE REACTION CATALYZED BY EXTRAMITOCHONDRIAL MALIC ENZYME FROM RAT SKELETAL MUSCLE

The decarboxylation rate was measured at 30°C in the medium (final volume 1 ml) containing: 50 mM Tris-HCl (pH 7.2)/20 mM imidazole-HCl (pH 7.2)/2.5 mM L-Malate/0.5 mM NADP/1 mM $MnCl_2$/0.4 μg malic enzyme. The rate of reductive carboxylation of pyruvate was measured in the medium (final volume 1 ml) containing: 50 mM Tris-HCl (pH 7.2)/20 mM imidazole-HCl (pH 7.2)/10 mM pyruvate/0.15 mM NADPH/1 mM $MnCl_2$/0.8 μg malic enzyme and $KHCO_3$ at concentration indicated in the Table. The rates of carboxylation were verified by measuring the malate present at the end of the reaction.

	Reaction rate	
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$	% of forward reaction
Decarboxylation	25.2	
Carboxylation $KHCO_3$, 5 mM	5.3	21
$KHCO_3$, 10 mM	9.0	35.7
$KHCO_3$, 25 mM	15.5	61.5
$KHCO_3$, 50 mM	20.0	79.4

point of the extramitochondrial malic enzyme from pig heart was at pH 5.1.

Reversibility of the reaction catalyzed by extramitochondrial malic enzyme from rat skeletal muscle. We have shown recently [5] that NADP-linked malic enzyme from rat skeletal muscle mitochondria catalyzes the reductive carboxylation of pyruvate at a relatively low rate as compared to the forward reaction. The results of experiments designed to show the ratio of decarboxylation to carboxylation catalyzed by extramitochondrial malic enzyme from rat skeletal muscle are shown in Table II. It is evident that the reaction catalyzed by extramitochondrial malic enzyme from rat skeletal muscle is readily reversible. The rate of pyruvate carboxylation in the presence of high concentration of bicarbonate can reach about 80% of the forward reaction. The carboxylation of pyruvate was documented both by observing the disappearance of NADPH and by enzymic determination of malate after reaction had been completed. In each experiment the oxidation of NADPH was accompanied by an equivalent amount of malate formed.

The dependence of activity on pH. Fig. 2 shows a comparison of the effect of pH on malic enzyme activity in both directions. The optimum pH for the carboxylation reaction was 7.1, both in the presence of 2.5 mM and 10 mM pyruvate (Fig. 2a). The sensitivity of the malic enzyme activity to the changes of pH in the case of decarboxylation direction varied with malate concentration (Fig. 2b). With malate concentrations of 0.05, 0.1, 1 and 10 mM the respective optima were about pH 6.6, 6.9, 7.5 and 7.9 (Fig. 2b). The Michaelis constant for malate also varied with pH (data not shown); the values calculated from Eadie-Hofstee plots increased with the increase of pH. As shown previously [5] the mitochondrial malic enzyme from rate skeletal muscle shows a sigmoid-shaped substrate saturation curve and this characteristic has been accentuated at higher pH values. In the case of extramitochondrial malic enzyme from rat skeletal muscle no evidence of sigmoidicity at any pH was observed. At pH values lower than 7.0 the enzyme was inhibited by high (greater than 1 mM) malate concentrations. A similar effect has been observed with bacterial [21] and plant [22, 23] malic enzyme.

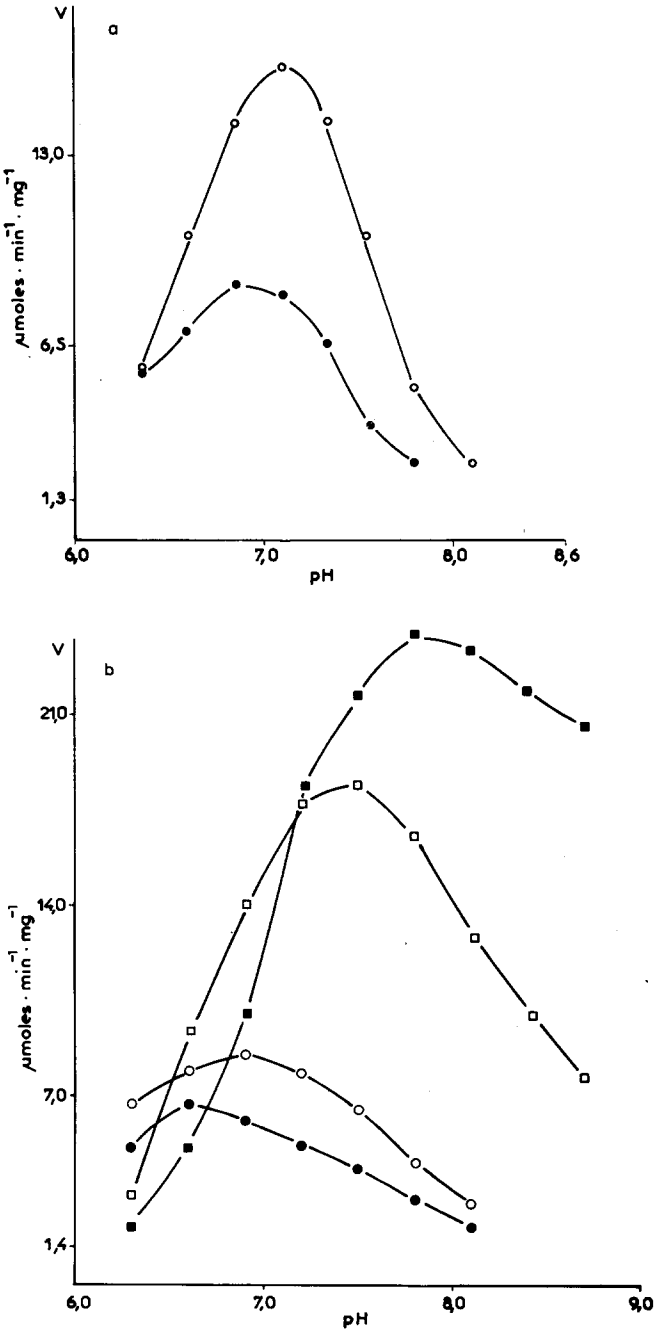

Fig. 2. a, The effect of pH on reductive carboxylation of pyruvate catalyzed by extramitochondrial malic enzyme. Reaction was carried out at 30°C in a medium (final volume 1 ml) containing: 50 mM Tris-HCl/20 mM imidazole-HCl/0.2 mM NADPH/1 mM $MnCl_2$/25 mM $KHCO_3$/2.5 mM pyruvate (●———●) or 10 mM pyruvate (○———○). b. The effect of pH on oxidative decarboxylation of malate catalyzed by extramitochondrial malic enzyme. Reaction was carried out at 30°C in the medium (final volume 1 ml) containing: 50 mM Tris-HCl/20 mM imidazole-HCl/0.5 mM NADP/1 mM $MnCl_2$/0.05 mM L-malate (●———●); 0.1 mM L-malate (○———○); 1 mM L-malate (□———□); 10 mM L-malate (■———■).

TABLE III

KINETIC CONSTANTS FOR THE EXTRAMITOCHONDRIAL MALIC ENZYME FROM RAT SKELETAL MUSCLE

Reaction rates for decarboxylation were measured at pH 7.2 in a medium containing: 20 mM Tris-HCl/20 mM imidazole-HCl/10 mM L-malate/0.5 mM NADP/1 mM $MnCl_2$/0.4 μg enzyme. The rates of reductive carboxylation were measured at pH 7.2 in a medium containing: 50 mM Tris-HCl/20 mM imidazole-HCl/ 50 mM $KHCO_3$/10 mM pyruvate/0.15 mM NADPH/1 mM $MnCl_2$/0.8 μg enzyme. For estimation of the K_m all components required were present at the concentrations mentioned above, with a varied concentration of the component tested. The K_m values were calculated from Eadie-Hofstee plots.

Decarboxylation		Carboxylation	
K_m(malate)	0.125 mM	K_m(pyruvate)	4.0 mM
K_m(NADP)	11 μM	K_m(NADPH)	6.6 μM
K_m($MnCl_2$)	9.5 μM	K_m($KHCO_3$)	24.0 mM

Kinetic parameters. To characterize further the extramitochondrial malic enzyme from rat skeletal muscle, some kinetic measurements were performed at pH 7.2, both for carboxylation and decarboxylation reaction. Table III summarizes apparent K_m values for different substrates. The K_m values presented do not differ essentially from the K_m values reported for extramitochondrial malic enzyme from other sources.

It has been reported that malic enzyme from pigeon liver [13] and other sources [6,24] is capable of catalyzing decarboxylation of oxaloacetate at pH 4.5, and that this reaction is stimulated by NADP. In our experiments the extramitochondrial malic enzyme from rat skeletal muscle catalyzed the decarboxylation of oxaloacetate (in conditions described under Materials and Meth-

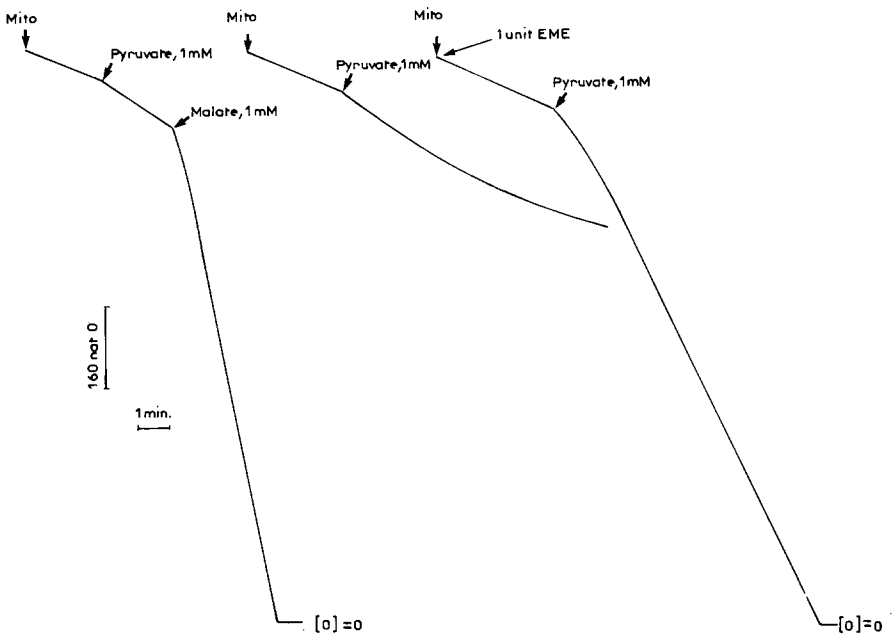

Fig. 3. Effect of pyruvate carboxylation by extramitochondrial malic enzyme on pyruvate oxidation by isolated rat skeletal muscle mitochondria. For experimental conditions see Table IV.

TABLE IV

EFFECT OF PYRUVATE CARBOXYLATION BY EXTRAMITOCHONDRIAL MALIC ENZYME ON PYRUVATE OXIDATION BY ISOLATED SKELETAL MUSCLE MITOCHONDRIA

Respiration was measured with a Clark oxygen electrode at 30°C in 2.5 ml medium containing: 15 mM KCl/50 mM Tris-HCl (pH 7.1)/10 mM $MgSO_4$/1 μM CCCP/20 mM $KHCO_3$/0.5 mM NADPH/2.2 mg mitochondrial protein. 1 unit of malic enzyme is defined as that amount catalyzing the reduction of 1 μmol NADP in the presence of 10 mM L-malate at pH 7.8 and 30°C. Values presented are means of two independent experiments. EME, extramitochondrial malic enzyme.

Conditions		Oxygen uptake natoms · min^{-1} per mg mitochondrial protein
Pyruvate (1 mM)		11.6
Malate (1 mM)		10.5
Pyruvate (1 mM)	+ Malate (1 mM)	146.8
Pyruvate (1 mM)	+ EME 0.03 unit	17.6
	+ EME 0.08 unit	31.3
	+ EME 0.16 unit	51.5
	+ EME 0.33 unit	64.1
	+ EME 1.0 unit	79.6
Pyruvate (1 mM)	+ EME 1.0 unit	
	− NADPH	11.6
	− $MgSO_4$	20.9
	− $KHCO_3$	17.4

ods) at a rate approaching about 10% of the rate of oxidative decarboxylation of malate. No activity was observed when Mn^{2+} was omitted. When experiments were carried out exactly under conditions described by Hsu and Lardy [13], NADP failed to affect the oxaloacetate decarboxylase activity.

As the carboxylation of pyruvate to malate could be catalyzed by extramitochondrial malic enzyme, the possibility was considered that the enzyme is involved in the anaplerotic maintenance of citrate cycle intermediates. In order to check this it was necessary to demonstrate that pyruvate carboxylation can provide malate, which in the mitochondria would undergo conversion to oxaloacetate required for condensation with acetyl-Co formed by oxidative decarboxylation of pyruvate (or by some other way from different precursors). Rat skeletal muscle mitochondria prepared as described previously [10] were able to oxidize pyruvate at a low rate in the absence of added malate. When malate was present in the incubation medium the amount of oxygen uptake during oxidation of pyruvate was increased due to the entry into the citric acid cycle. As can be seen in Fig. 3 when bicarbonate, NADPH and purified extramitochondrial malic enzyme were added to mitochondria oxidizing pyruvate (in the presence of CCCP), an increase of oxygen uptake was observed. The conditions necessary for the increase of oxygen uptake are more definitely shown in Table IV. The stimulation of pyruvate oxidation was completely dependent on the presence of extramitochondrial malic enzyme, NADPH, bicarbonate and Mg^{2+}. Omission of any one of these, resulted in a lack of stimulation of pyruvate oxidation. It thus appears that under conditions in which extramitochondrial malic enzyme is active, malate (formed from pyruvate) is delivered to the citric acid cycle at a rate sufficient to maintain rapid oxidation of pyruvate. In order to estimate the amount of extramitochondrial malic enzyme required to pro-

duce enough malate from pyruvate, the experiments presented in Table IV were carried out. As may be seen stimulation was already evident with a rather small amount of malic enzyme. At this point it is worth mentioning that from 1 g of a mixed type of muscle used in these experiments, we are able to obtain about 2 mg mitochondrial protein and about 0.2 unit extramitochondrial malic enzyme. It therefore seems probable that the ratio of the amount of mitochondria used in our experiments to that of extramitochondrial malic enzyme is close to physiological. Nevertheless, we recognize that this estimate does not include mitochondrial protein which we were losing during the preparative procedure.

Discussion

The purpose of the present investigation was to provide evidence that purified extramitochondrial malic enzyme from rat skeletal muscle is able to catalyze the reductive carboxylation of pyruvate to malate, as a possible pathway involved in the anaplerotic maintenance of Krebs cycle intermediates. Although the activity of malic enzyme in mammalian skeletal muscle has been reported before [3,4,25], to our knowledge no detailed studies concerning its purification and properties have been described. The activity of extramitochondrial malic enzyme in postmitochondrial supernatant is in the range 6—10 nmol · min^{-1} per mg protein. This is comparable to the activity of 3 nmol · min^{-1} per mg of cytosol protein reported by Bartholome et al. [18] in pig heart. The purification procedure employed for this enzyme, the general outline of which was used for the isolation of extramitochondrial malic enzyme from other sources, yields homogeneous protein as judged by gel electrophoresis. The isolated enzyme from rat skeletal muscle appears to be similar to the extramitochondrial malic enzyme from other sources as far as its molecular weight, subunit structure and kinetic properties are concerned. However, there are some differences between mitochondrial and extramitochondrial malic enzyme from rat skeletal muscle [5]. The most striking differences between the two enzymes is the ratio of the abilities to catalyze the decarboxylation and carboxylation reactions. The reaction catalyzed by the mitochondrial malic enzyme shows negligible reversibility, since the reaction of carboxylation reaches only 15% of the decarboxylation reaction [5]. On the other hand the reaction catalyzed by extramitochondrial malic enzyme appears to be readily reversible, as this enzyme catalyzed the reductive carboxylation of pyruvate at a rate approaching about 80% that of decarboxylation (measured at the same conditions). This suggests that mitochondrial malic enzyme from rat skeletal muscle is operating preferentially in the decarboxylation direction. It is in accordance with the suggestions that mitochondrial malic enzyme plays an essential role in the conversion of tricarboxylic cycle intermediates to pyruvate [1,2,5,6]. On the other hand, extramitochondrial malic enzyme might operate in the carboxylation direction to produce malate from pyruvate and bicarbonate.

It therefore appears, that one function of extramitochondrial malic enzyme might be to replenish the dicarboxylic acid pool in order to maintain the operation of the citric acid cycle during fatty acid oxidation in skeletal muscle. Another possibility is that the extramitochondrial malic enzyme in skeletal

muscle is one of the important enzymes of the glycogen from lactate biosynthesis as suggested by Bendall and Taylor [26]. The recent study of Hermansen and Vaage [27,28] confirmed the findings of Bendall and Taylor [26] showing that glycogen can be formed from lactate in human skeletal muscle during recovery after maximal exercise.

Acknowledgements

I am indebted to Professor M. Żydowo for a critical reading and discussion of the manuscript. I am grateful to Dr. P.W.D. Ścisłowski for help in performing some experiments and Dr. J. Spychała for performing the gel electrophoresis. This work was supported by the Polish Academy of Sciences within the project II.1.2.6.

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