Glyoxylate cycle enzymes are present in liver peroxisomes of alloxan-treated rats

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Abstract Key enzymes of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS), have been detected in the liver of alloxan-treated rats. The activity of ICL in rat liver was 0.040 μmol/min/mg protein and the activity of MS was 0.022 μmol/min/mg protein. These enzymes were associated with the peroxisomal fraction. The activities of citrate synthase, malate synthase and malate dehydrogenase detected in the peroxisomal fraction were also increased by alloxan treatment. Isocitrate lyase was partially purified and displayed catalytic and regulatory properties similar to those of the enzyme isolated from the liver of starved rats (Popov, V.N. et al. (1996) FEBS Lett. 391, 87–90).

Key words: Glyoxylate cycle; Isocitrate lyase; Malate synthase; Induction; Alloxan; Diabetes

1. Introduction

Glyoxylate cycle (GC) enzymes have been found in the larvae of lower invertebrates, where this cycle facilitates the utilization of stored fats for gluconeogenesis during growth [1,2]. Some data pointing to the possibility of glyoxylate cycle operation have also been published for vertebrates [3,4]. We have shown previously that key enzymes of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS) can be detected in liver extracts of starved rats. We have determined the activities of these enzymes, purified and characterized them. These data established the existence of these key enzymes of the glyoxylate cycle in mammalian tissue [5,6]. It has provided the biochemical basis for the physiological phenomena of de novo glycogen synthesis in rat liver upon starvation [7,8]. The time course of isocitrate lyase and malate synthase induction correlated perfectly with the kinetics of glycogen synthesis, and the mechanism of this synthesis may now be discussed in terms of carbon flow from acetyl-CoA. We suggested that in mammalian tissue an increase in glycogen synthesis upon food starvation from acetyl-CoA could be due to the glyoxylate cycle, similar to carbohydrate synthesis in bacterial or plant cells.

The glyoxylate cycle was originally discovered by Kornberg and Krebs in bacteria grown on acetate [9]. The cycle couples the metabolism of two-carbon compounds to gluconeogenesis. Malate synthase and isocitrate lyase were recognized as pathway-specific enzymes, while the other enzymes involved were in common with the mitochondrial TCA cycle. Later, the glyoxylate cycle was also detected in germinating seeds of plants [10]. It was shown to involve its own specific enzyme complement in the isolated glyoxysomes in addition to enzymes catalyzing fatty acid β-oxidation.

Subsequently, different types of microbodies were discovered in plant tissue. One of these types, named glyoxysomes, containing the enzymes of the glyoxylate cycle and of the β-oxidation pathway, was detected in germinating fat-storing seeds [11]. Induction of the glyoxylate cycle by starvation is associated with the degradation of liver tissue, and it is an extreme physiological process. Participation of this pathway in fatty acids to carbohydrate conversion may be related to other situations, which are associated with the mobilization of fatty acids. One such physiologically occurring process is diabetes, which causes a decrease in carbohydrate concentration paralleled by intensive mobilization of fats [12]. Glycogen formation in the diabetic liver utilizing fatty acids as a source of carbon skeleton synthesis has also been shown [8]. Using the same experimental model of alloxan-induced diabetes, we investigated the possibility of induction of the glyoxylate cycle enzymes under these conditions. We report here the induction of glyoxylate cycle enzymes by alloxan treatment and describe the subcellular location of these enzymes, as well as some catalytic properties of the key enzyme of the cycle, isocitrate lyase.

2. Materials and methods

Three months old rats (*Rattus rattus* L.) fed with standard rat chow were injected with alloxan (200 mg per kg of weight in 0.9% NaCl solution). Control animals were treated with the isotonic NaCl solution. Induction of diabetes was monitored by measuring the glucose concentration in blood. In the control group the glucose concentration was 8.3 ± 1.1 mM. Alloxan injection induced an increase in blood glucose up to 18.7 ± 2.0 mM, seven days after injection.

Animals were anesthetized with ethyl ester and decapitated. The liver was excised and weighed, then 1 g of liver tissue was homogenized in 10 ml of 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA and 0.1% Triton X-100. The homogenate was centrifuged at 4000 × *g* for 5 min. The supernatant was used for enzyme analysis. When the supernatant was used for the preliminary isolation of organelles, Triton X-100 was omitted and 0.3 M sucrose was added to the medium. For organelle isolation, the supernatant was applied on top of a continuous sucrose gradient (25–57%) buffered with 50 mM Tris-HCl, containing 1 mM $MgCl_2$, 1 mM dithiothreitol and 0.5 mM EGTA. It was centrifuged to equilibrium for 2.5 h at 100 000 × *g* max. Two-ml fractions were collected, diluted up to 0.25 mM sucrose concentration and centrifuged 15 min at 20 000 × *g* max. The pellet was resuspended in 50 mM Tris-HCl buffer, containing 1 mM dithiothreitol, 1 mM $MgCl_2$ and 0.1% Triton X-100, and used for detection of enzyme activities.

Enzyme assays were performed at 25°C. Isocitrate lyase activity was

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Abbreviations: AH, aconitate hydratase; CS, citrate synthase; GC, glyoxylate cycle; ICL, isocitrate lyase; MDH, malate dehydrogenase; MS, malate synthase; PPAR, peroxisome proliferator-activated receptor

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measured using the method of Dixon and Kornberg [13]. The assay mixture of 1 ml consisted of 50 mM Tris-HCl, pH 7.5, 2 mM isocitrate, 5 mM $MgCl_2$ and 4 mM phenylhydrazine-HCl. The reaction was followed spectrophotometrically at 324 nm. An extinction coefficient of 1.67×10^4 M^{-1} cm^{-1} was used. Malate synthase activity was measured according to Hock and Beevers [14]. The assay mixture consisted of 50 mM Tris-HCl, pH 7.6, 0.15 mM acetyl-CoA, 2 mM glyoxylate, and 1 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid). The activity was measured at 412 nm using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. Catalase activity was determined according to Breidenbach et al. [15], and the activity of succinate dehydrogenase, fumarase and alcohol dehydrogenase according to Cooper and Beevers [16], and one unit (E.U.) of activity was defined as the conversion of 1 μmol of a substrate per min. Protein was measured according to Lowry et al. [17].

Isocitrate lyase was purified by the following procedure. Proteins of a crude extract (10 ml) were precipitated by ammonium sulfate (50–70% saturation), maintaining pH 7.5 by adding 0.1 M NaOH. Proteins were dissolved and desalted on a Sephadex G-25 column (15×1 cm). A test with Nessler reagent was used to control the complete removal of ammonium sulfate. Proteins were applied to a DEAE-cellulose 52 (Whatman, UK) column (20×2 cm) equilibrated with 10 mM Tris-HCl. Proteins were eluted by a step gradient, eluting first with 20 ml of 30 mM KCl and then with 40 mM KCl in column buffer. Fractions with isocitrate lyase activity were collected, dialyzed against the column buffer and applied to a Toyopearl HW-65 (Toyo-Soda, Japan) column (90×2 cm) equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 3 mM $MgCl_2$, 1 mM EDTA and 3 mM dithiothreitol. Elution was performed with the same buffer, and fractions containing isocitrate lyase activity were used for enzyme characterization.

3. Results

No isocitrate lyase or malate synthase activity was detected in crude extracts of livers isolated from non-treated animals. Injection of alloxan induced both ICL and MS activities. These activities became measurable in the liver of rats having a blood glucose concentration greater than 15 mM, i.e. with developed diabetes. The activities were detectable from 7 to 30 days after injection. Seven days after alloxan injection the specific activity of isocitrate lyase in crude extracts of liver tissue was 0.040, and of malate synthase 0.022 μmol/min/mg protein (Fig. 1). Determination of these enzymes in other tissues has shown that ICL and MS activities were present in rat kidney (Table 1). Determination of malate dehydrogenase, citrate synthase and aconitate hydratase activities also revealed their increase in livers of alloxan-treated rats (Fig. 1). Thus, the activity of glyoxylate cycle enzymes was detected only in the liver of rats with alloxan diabetes.

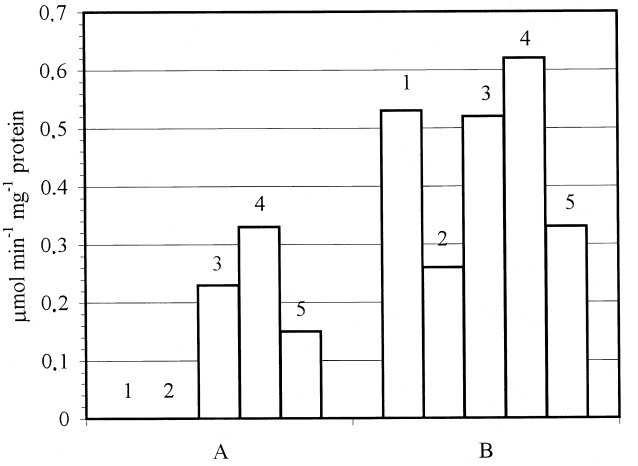

Fig. 1. Induction of glyoxylate cycle enzymes activities (μmol/min/mg protein) in liver of alloxan-treated rats. A: Control animals (0.9% NaCl injection). B: Seven days after alloxan injection (200 mg per kg weight); 1, isocitrate lyase; 2, malate synthase; 3, malate dehydrogenase; 4, citrate synthase; 5, aconitate hydratase.

To determine the intracellular localization of isocitrate lyase in rat liver tissue, an isopycnic centrifugation of a crude organelle extract (the supernatant after centrifugation at $4000\times g$ for 5 min) was performed. Catalase served as a marker for peroxisomes, succinate dehydrogenase and fumarase served as markers for mitochondria, and lactate dehydrogenase was a marker for the cytosolic fraction. Isocitrate lyase and malate synthase activities were confined to the fraction where the maximal catalase activity was revealed, i.e. to the fraction of specialized peroxisome-like organelles (Table 2). About 35% of total activity of ICL and MS was detected in the cytosol, but it corresponds to the same amount of catalase solubilized from ruptured microbodies. Therefore it has to be supposed that isocitrate lyase and malate synthase activities are located in the peroxisomal fraction. Increasing activities of malate dehydrogenase (MDH), citrate synthase (CS) and aco-

Table 1
Tissue specificity of glyoxylate cycle key enzymes in rat (7 days after alloxan injection, 200 mg per kg rat weight), μmol/min/g fresh weight

	Liver	Heart	Muscle	Kidney	Blood	Brain
Isocitrate lyase	2.6	No	No	0.94	No	No
Malate synthase	1.4	No	No	0.60	No	No

Table 2
Subcellular location of glyoxylate cycle enzymes (μmol/min) in control and alloxan-treated rats (7 days after injection of 200 mg/kg weight of alloxan)

	Control			Alloxan diabetes		
	Cytosol	Mitochondria	Peroxisomes	Cytosol	Mitochondria	Peroxisomes
ICL	0	0	0	0.136	0.072	0.325
MS	0	0	0	0.091	0.041	0.124
CS	0.08	0.125	0.025	0.131	0.200	0.190
MDH	0.12	0.185	0.029	0.182	0.245	0.200
AH	0.052	0.08	0.018	0.085	0.060	0.181
Protein (mg)	6.45	5.51	3.24	6.57	5.7	3.4

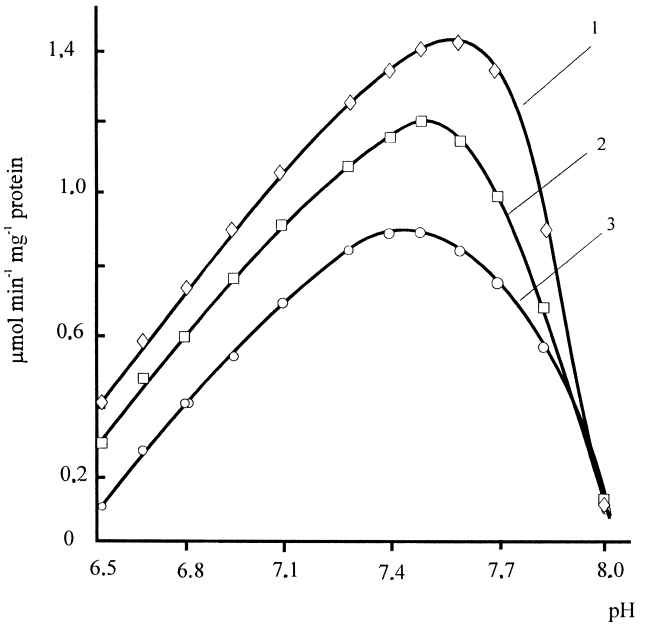

Fig. 2. pH dependence of isocitrate lyase activity from liver of alloxan-treated rats. 1, Tris-HCl, 50 mM; 2, Na_2HPO_4/NaH_2PO_4, 50 mM; 3, Tris-HEPES, 20 mM.

nitate hydratase (AH) were also connected with the peroxisomal fraction. Whereas MDH, CS and AH activities were increased in the cytosol and mitochondrial fractions by only 1.2–2.0-fold, the activities in microbodies increased 7–10-fold.

For preliminary enzyme characterization, isocitrate lyase was purified 108.7-fold by ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose and gel-chromatography on Toyopearl HW-65 (Table 3). The specific activity of the purified enzyme was 8.7 μmol/min/mg protein. Rat liver isocitrate lyase showed a Michaelis kinetics with a K_m (isocitrate) = 68 μM and a pronounced pH optimum at pH 7.4–7.6 (Fig. 2).

The regulatory properties of ICL from liver of alloxan-treated rats were studied using the method of Dixon [18]. It was shown that glucose-6-phosphate and glucose-1-phosphate are the competitive inhibitors of ICL with K_i of 1.25 and 1.6 mM, respectively. Citrate inhibits ICL with a K_i of 0.95 mM; an inhibition of isocitrate lyase by the reaction product, succinate, was discovered (K_i=1.75 mM).

4. Discussion

In this study the presence of key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, was found in liver extracts of alloxan-treated rats. Induction of these enzymes correlated with the increase of glucose concentration in blood, i.e. with the induction of alloxan diabetes. The increase in activities of other glyoxylate cycle enzymes was also detected. Thus, we can conclude that alloxan diabetes causes the induction of complete glyoxylate cycle. The possible role of glyoxylate metabolic pathway in diabetic rats may be related to mobilization of fatty acids. The discovery in 1976 by Lazarow and De Duve of the peroxisomal β-oxidation of fatty acids in rats substantiates the possibility of acetyl-CoA formation in these organelles [19]. Operation of the glyoxylate cycle in peroxisomes would result in efficient succinate production which in turn can fuel gluconeogenesis in the rat liver. The microbodial localization of isocitrate lyase and malate synthase in rat liver is not surprising because glyoxylate cycle enzymes have often been detected in specialized microbodies (glyoxysomes) [11], and indications on different localization are rare [20].

It should be emphasized that we detected the rise in activities of all glyoxylate cycle enzymes recovered in the peroxisomal fraction. The presence of a complete glyoxylate cycle in peroxisomes would liberate acetyl-CoA formation and utilization from mitochondrial control.

We have shown earlier that the glyoxylate cycle is induced in the liver of starved rats [5,6]. The properties of purified enzymes isolated from the liver of alloxan-treated rats corresponded to previously published data for isocitrate lyase isolated from the liver of starved rats. These enzymes have similar specific activities, catalytic and regulatory properties. However, inhibition by citrate and succinate was not reported earlier. This may be related to the physiological significance of this phenomenon, since mobilization of fatty acids is necessary for supporting the energy balance and carbohydrate level during diabetes, and to the regulation of further metabolism of succinate.

Diabetes and starvation usually cause a rise in fatty acid concentration in blood and liver tissues [21]. This fact may be related to the mechanism of glyoxylate cycle induction through the operation of a peroxisome proliferator-activated receptor (PPAR). Activation of this receptor causes the expression of key enzymes of fatty acid metabolism [22] and it is possible that GC expression is regulated by a similar mechanism. It is interesting to note that Wilson and coauthors [23] showed one of the antidiabetics, thiazolidinedione, to influence PPAR and to activate peroxisomal proliferation.

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Table 3
Purification of isocitrate lyase from liver of alloxan-treated rats

Purification step	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg protein)	Yield (%)	Purification factor (-fold)
Crude extract	20.5	260.0	0.08	100	1
Ammonium sulfate fractionation (50–70% of saturation)	12.3	54.6	0.23	60.0	2.8
DEAE-Toyopearl	6.7	2.5	2.68	32.6	33.5
Toyopearl HW-65	3.1	0.35	8.70	15.1	108.7

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