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Induction of glyoxylate cycle enzymes in rat liver upon food starvation

Vasily N. Popov[a], Abir U. Igamberdiev[a,*], Claus Schnarrenberger[b,**], Sergei V. Volvenkin[a]

[a]*Department of Plant Physiology and Biochemistry, Voronezh State University, Voronezh 394693, Russian Federation*
[b]*Institute for Plant Physiology and Microbiology, Freie Universität Berlin, Königin-Luise-Str. 12-16a, D-14195 Berlin, Germany*

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Abstract The key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, have been detected in liver of food-starved rats. Activities became measurable 3 days and peaked 5 days after the beginning of starvation. Both enzymes were found in the peroxisomal cell fraction after organelle fractionation by isopycnic centrifugation. Isocitrate lyase was purified 112-fold by ammonium sulfate precipitation, and chromotography on DEAE-cellulose and Toyopearl HW-65. The specific activity of the purified enzyme was 9.0 units per mg protein. The K_m(isocitrate) was 68 μM and the pH optimum was at pH 7.4. Malate synthase was enriched 4-fold by ammonium sulfate precipitation. The enzyme had a K_m(acetyl-CoA) of 0.2 μM, a K_m(glyoxylate) of 3 mM and a pH optimum of 7.6.

Key words: Isocitrate lyase; Malate synthase; Rat liver; Glyoxylate cycle

1. Introduction

The glyoxylate cycle was originally discovered by Krebs and Kornberg [1] in bacteria growing on acetate. The cycle connects the metabolism of two-carbon compounds with gluconeogenesis. Malate synthase and isocitrate lyase were recognized as pathway-specific enzyme activities while the remaining enzyme activities were in common with activities of the TCA cycle. The glyoxylate cycle was later also detected in germinating seeds of plants [2]. The function of the glyoxylate cycle was recognized as part of the intensive fat-to-carbohydrate interconversion during seed germination. Plants contain a full complement of glyoxylate cycle enzymes in the glyoxysomes, besides the enzymic sequence of β-oxidation to yield acetyl-CoA from fatty acid degradation [3].

Glyoxylate cycle activities have also been discovered in some animal tissues such as larvae of nematodes, urinary bladder epithelium cell layer of *Buffus marinus* [4], and liver of chicken which, after suffering from vitamin D deficiency, was replenished with vitamin D [5]. In mammals, glyoxylate cycle activities have only been found in cartilage tissue of the epiphyseal growth plate of rats [6]. Other occurrences have not yet been reported, possibly because the proper conditions for the induction of such activites are unknown.

Recently, however, it was observed in histological studies [7] that glycogen in rat liver first decreased upon food starvation and, thereafter, increased again. According to label incorporation into radioactive fatty acids, Lebkova [8] proposed a mechanism of secondary glycogen production, possibly by conversion of fat to carbohydrates. Although other reactions could also account for glycogen synthesis in the liver of starved rats, proof of the presence or absence of glyoxylate activities in liver of starved rats would be of prime interest because this would document the induction of the glyoxylate cycle in mammalian rat liver. Here, we report the discovery of the key enzymes of the glyoxylate cycle in liver of starved rats as support for the suggestion that prolonged food starvation leads to glycogen formation in liver. We have characterized the activities of partially purified malate synthase and isocitrate lyase and demonstrate their compartmentalization in the peroxisomal cell fraction.

2. Materials and methods

3-month-old rats (*Rattus rattus* L.) were fed regularly and then transferred to food starvation and excessive water supply for up to 7 days. Animals were anaesthetized with diethyl ether before decapitation. 1 g of liver was excised and homogenized in 10 ml of 0.4 M sucrose, 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA using a mortar and pestle for preparation of crude homogenates. Centrifugation was at $4000\times g$ for 5 min. The supernatant was used for enzyme assays.

For preparation of cell organelles, 0.1% Triton X-100 was omitted from the grinding medium, 0.3 M sucrose being included instead. After the preliminary sedimentation of unruptured tissue fragments, nuclei, and cell debris, the supernatant was applied to a continuous sucrose gradient (25–57%) buffered with 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM EDTA. Particles were centrifuged to equilibrium for 2.5 h at $100\,000\times g$. 2-ml fractions were collected, diluted to 0.25 mM sucrose and centrifuged for 15 min at $20\,000\times g$. The pellets were resuspended, each in 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 0.1% Triton X-100, and used for enzyme measurements.

Enzyme assays were performed at 25°C and followed photometrically. Isocitrate lyase was determined according to the method of Dixon and Kornberg [9]. The assay mixture of 1 ml volume consisted of 50 mM Tris-HCl, pH 7.5, 0.5 mM isocitrate, 5 mM $MgCl_2$, and 4 mM phenylhydrazine-HCl. The reaction was followed at 324 nm. An extinction coefficient of 1.67×10^4 M^{-1} cm^{-1} was used. Malate synthase was measured according to Hock and Beevers [10]. 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′-dithiobis(2-nitrobenzoic acid). The activity was followed at 412 nm. The extinction coefficient was 1.36×10^4 M^{-1} cm^{-1}. Catalase was determined according to Breidenbach et al. [11], succinate dehydrogenase, fumarase, and alcohol dehydrogenase as described by Cooper and Beevers [12], and protein according to Lowry et al. [13]. One unit of activity was defined as the conversion of 1 μmol of substrate per min.

Isocitrate lyase was purified via the following procedure. Proteins of a crude extract (10 ml) were precipitated using ammonium sulfate (50–70% saturation), maintaining the pH at 7.5 with 0.1 M NaOH. Proteins were dissolved and desalted through a Sephadex G-25 column (15×1 cm). A test with Nessler reagent was used to control the complete removal of ammonium sulfate. Finally, proteins were applied to a DEAE-cellulose 52 (Whatman, UK) column (20×2 cm) equilibrated with 10 mM Tris-HCl, pH 7.5. Proteins were eluted with a stepwise gradient, eluting first with 20 ml of 30 mM KCl and then with 40 mM KCl in column buffer. Fractions with isocitrate

*Corresponding author. Fax: (7)-(073)-2-7689755.
E-mail: root@bc.vucnit.voronezh.su

**For fast correspondence: Fax: (49) (30)-838-4313.
E-mail: schnarre@fub46.zedat.fu-berlin.de

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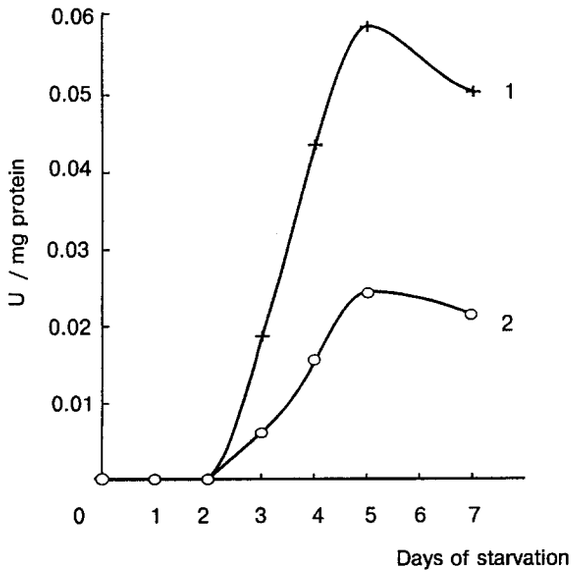

Fig. 1. Changes in the activities of isocitrate lyase (1) and malate synthase (2) in liver of starved rats.

lyase activity were collected, dialyzed against column buffer, and applied to gel chromatography on a Toyopearl HW-65 (Toyo-Soda, Japan) column (90×2 cm) equilibrated with 25 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$ 1 mM EDTA, and 3 mM dithiothreitol. Elution was performed with the same buffer. Fractions with isocitrate lyase activity were used for enzyme characterization.

Malate synthase was enriched by 0–20% ammonium sulfate fractionation and by desalting on a Sephadex G-25 column as described above.

3. Results

When isocitrate lyase and malate synthase were monitored in crude extracts of rat liver (Fig. 1), no activity was detectable in non-starved animals. However, 3 days after food starvation, the activity of both enzymes began to emerge and rose considerably, reaching a maximum at day 5. After 7 days, the experiments had to be discontinued because the animals became too unstable physically. The specific activity of isocitrate lyase and malate synthase in crude extracts of liver tissue was 0.060 and 0.025 units/mg protein, respectively.

In order to determine the intracellular localization of isocitrate lyase and malate synthase in rat liver, isopycnic centrifugation of a crude organelle extract was performed and the activity of catalase as a marker for peroxisomes, of succinate dehydrogenase and fumarase as markers for mitochondria, and of alcohol dehydrogenase as a marker for the non-particulate cell fraction was determined. Isocitrate lyase and malate synthase (Fig. 2) were confined to the fraction with the highest particulate catalase activity. Therefore, isocitrate lyase and malate synthase are located in the peroxisomes.

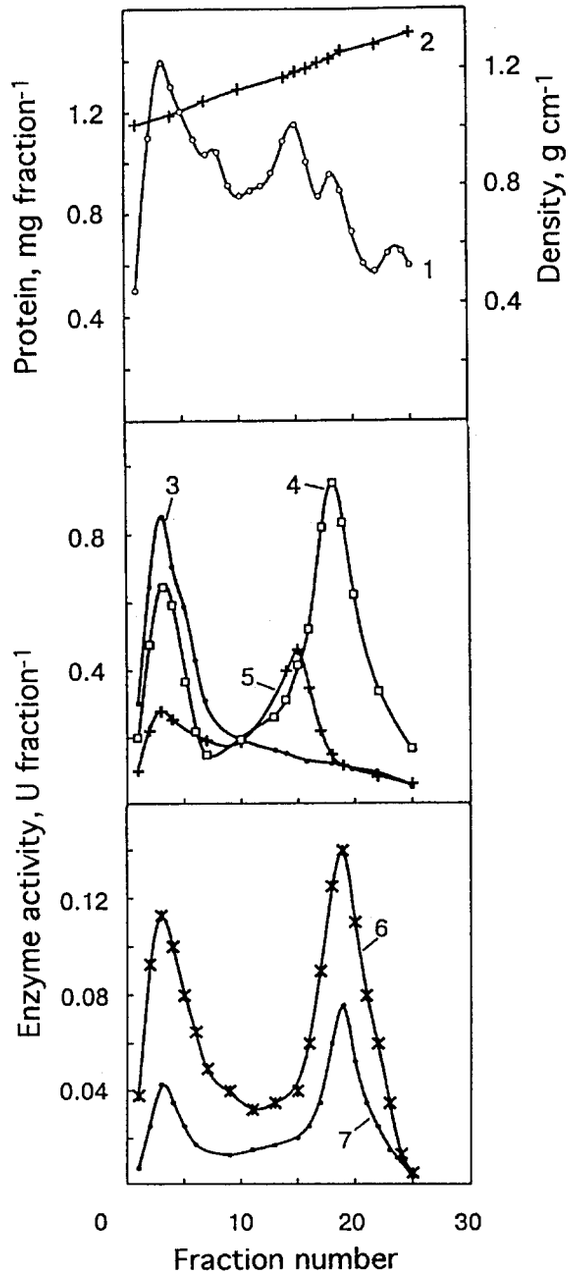

Fig. 2. Profile of isocitrate lyase and malate synthase and of marker enzyme activities for cell organelles separated by isopycnic centrifugation in a sucrose gradient. 1, protein; 2, gradient density; 3, alcohol dehydrogenase; 4, catalase; 5, fumarase; 6, isocitrate lyase; 7, malate synthase.

For preliminary enzyme characterization, isocitrate lyase was purified 112-fold by ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose, and gel chromatography on Toyopearl HW-65 (Table 1). The specific

Table 1
Partial purification of isocitrate from rat liver

Purification step	Activity (units)	Protein (mg)	Spec. act. (units/mg)	Yield (%)	Purification (-fold)
Crude extract	24.3	304	0.08	100	1
50–70% $(NH_4)_2SO_4$	12.6	72	0.18	51.8	2.3
DEAE-cellulose	6.5	2.1	3.1	26.7	38.8
Toyopearl HW-65	2.7	0.3	9.0	11.1	12.5

activity of the purified enzyme was 9.0 units per mg protein. Rat liver isocitrate lyase demonstrated Michaelis-Menten kinetics with a K_m(isocitrate) of 68 μM (Fig. 3) and a pronounced pH optimum at pH 7.4 (Fig. 4). ADP at a concentration of 1 mM activated isocitrate lyase by about 40%.

Malate synthase was enriched 4-fold by ammonium sulfate precipitation with a yield of 48% and a specific activity of 0.076 units per mg protein, however, the activity was lost during subsequent chromatography on DEAE-cellulose. The enzyme has a K_m(acetyl-CoA) of 0.2 μM and a K_m(glyoxylate) of 3.0 mM. The pH optimum was 7.6 (Fig. 4).

4. Discussion

In the present investigation the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, have been demonstrated in liver extracts of food-starved rats. To date, there has only been scarce evidence for the glyoxylate cycle in animals, except in larvae of lower invertebrates in which it serves for utilization of stored fats for gluconeogenesis during growth [14], in some detailed instances in vertebrates [4,5], and in cartilage tissue of the epiphyseal growth plate of rats [6]. The reason for the present finding was that we used starved rats and analyzed these enzymes in liver tissues. This tissue, together with food starvation, provides all physiological phenomena of the de novo synthesis of glycogen [7,8]. The time course of isocitrate lyase and malate synthase correlates perfectly with that of glycogen synthesis. As a consequence, the mechanism of glycogen synthesis upon food starvation should now be discussed in terms of carbon flow from acetyl-CoA.

The properties of purified isocitrate lyase and malate synthase correspond to previously reported data for these enzymes from other sources, e.g. plants and microorganisms [15,16]. However, ADP stimulation has not been reported previously. It may correspond to the physiological significance of this phenomenon, as mobilization of fatty acids is necessary for supporting energy balance and carbohydrate levels during starvation. The peroxisomal localization of isocitrate lyase and malate synthase in rat liver is usual because glyoxylate cycle enzymes have been detected in specialized microbodies (glyoxysomes) [3], and indications on other localizations are rare [17].

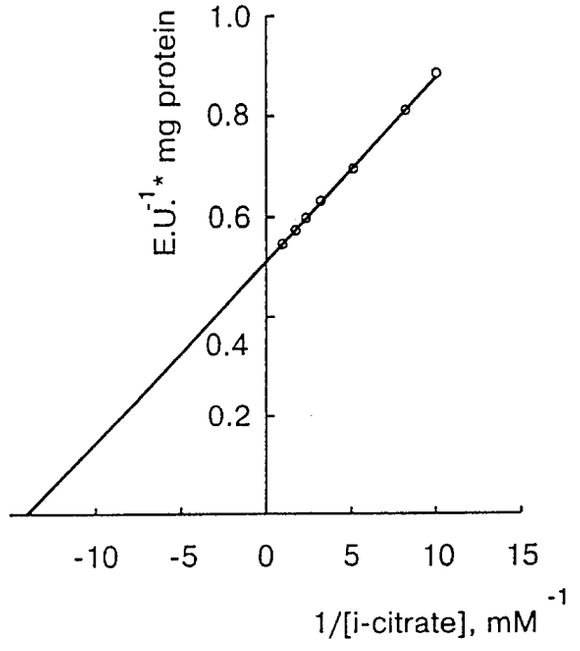

Fig. 3. Enzyme kinetics of isocitrate lyase for isocitrate.

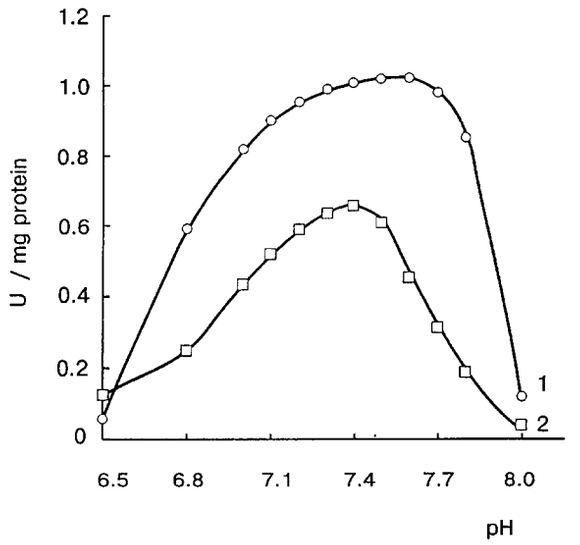

Fig. 4. pH dependence of purified isocitrate lyase (1) and malate synthase (2) from liver of starved rats.

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References

[1] Krebs, H. and Kornberg, H.L. (1957) Nature 179, 988–991.
[2] Kornberg, H.L. and Beevers, H. (1957) Biochim. Biophys. Acta 26, 531–537.
[3] Beevers, H. (1979) Annu. Rev. Plant Physiol. 30, 159–193.
[4] Goodman, D.B.P., Davis, W.L. and Jones, R.G. (1980) Proc. Natl. Acad. Sci. USA 77, 1521–1525.
[5] Davis, W.L., Jones, R.G., Farmer G,R., Dickerson, T., Cortinas, E., Cooper, O.J., Crawford, L. and Goodman, D.B.P. (1990) Anat. Rec. 227, 271–284.
[6] Davis, W.L., Jones, R.G., Farmer, G.R., Matthews, J.L. and Goodman, D.B.P. (1989) Anat. Rec. 223, 357–362.
[7] Lebkova, N.P. (1985) Russ. J. Cytol. 27, 157–163.
[8] Lebkova, N.P. (1984) Bull. Exp. Biol. Med. (in Russian) 12, 73–76.
[9] Dixon, G.H. and Kornberg, H.L. (1959) Biochem. J. 72, 3–13.
[10] Hock, B. and Beevers, H. (1966) Z. Pflanzenphysiol. 55, 405–414.
[11] Breidenbach, R.W., Kahn, A. and Beevers, H. (1968) Plant Physiol. 43, 703–713.
[12] Cooper, T.G. and Beevers, H. (1969) J. Biol. Chem. 244, 3507–3513.
[13] Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) J. Biol. Chem. 194, 265–275.
[14] Rubin, H. and Trelease, R.N. (1976) J. Cell. Biol. 70, 374–383.
[15] Igamberdiev, A.U. and Zemlyanukhin, A.A. (1987) Biochemistry (Moscow) 52, 1286–1293.
[16] Tahama, H., Shinoyama, H. and Ando, A. (1990) Agric. Biol. Chem. 54, 3177–3183.
[17] Igamberdiev, A.U., Zemlyanukhin, A.A. and Meshcheryakova, I.V. (1986) Sov. Plant Physiol. 33, 852–858.