THE ATP-DEPENDENT REDUCTIVE CARBOXYLATION OF 2-OXOGLUTARATE USING CYTOSOL FROM RAT LIVER[†]

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Abstract

An enzyme system (isocitrate synthase) catalysing the conversion of 2-oxoglutarate to isocitrate has been detected in and partially purified from rat liver. The activity of this enzyme system is dependent on the presence of Mg^{2+}, ATP, HCO_3^- and NADPH.

The major proportion of the isocitrate synthase was found in the cytosol and its level of activity was related to the nutritional state of the animal, undergoing changes parallel to those observed in the rate of lipogenesis. During purification, the activity of the isocitrate synthase system increased relative to that of isocitrate dehydrogenase ($NADP^+$).

The labelling patterns obtained in studies on the incorporation of specifically labelled [^{14}C]glutamate into fatty acids in mammalian liver have indicated that glutamate was converted to fatty acids by a pathway in the cytoplasm, analogous to a reversal of part of the citric acid cycle. The postulated sequence of events involves the conversion of glutamate to 2-oxoglutarate, which is assumed to undergo reductive carboxylation to isocitrate, isomerization of isocitrate to citrate and cleavage of citrate to oxaloacetate and acetyl CoA (1,2). Evidence is available to suggest that this so-called "oxoglutarate shunt" also operates in adipose tissue (3,4), mammary gland (5) and brain (6,7).

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In this pathway, the reductive carboxylation of oxoglutarate to isocitrate has been assumed to be catalysed by the NADP-dependent isocitrate dehydrogenase (EC.1.1.1.42). However, in this communication we present evidence for the existence in rat liver cytosol of an enzyme system which catalyses the following reaction sequence:-

$$\text{2-oxoglutarate} + MgATP^{2-} + HCO_3^- \rightarrow \text{oxalosuccinate} + MgADP^- + Pi \quad \ldots(i)$$

$$\text{oxalosuccinate} + NADPH + H^+ \rightarrow \text{isocitrate} + NADP^+ \quad \ldots(ii)$$

independently of isocitrate dehydrogenase.

MATERIALS AND METHODS

2-Oxoglutarate carboxylase activity, - Routine assays were carried out in a total volume of 0.25 ml containing, 100 mM N-ethylmorpholine, pH 7.5; 2 mM ATP; 4 mM $MgCl_2$; 10 mM $NaH^{14}CO_3$ (0.25 µCi per µmole); 0.1 mM NADPH; 3.5 mM oxoglutarate and enzyme. All incubations were carried out at 30°C. In this assay, ATP was omitted from the controls. The reaction was stopped by the addition of 50 µl of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. Denatured protein was removed by centrifuging and aliquots of the supernatant solution were spotted on Whatman 3 MM filter paper squares, dried and the acid-stable radioactivity determined.

Protein determination, - Protein concentrations were determined by the method of Warburg and Christian (8).

Isocitrate dehydrogenase (NADP) activity, - The enzyme activity was measured in tris buffer, pH 7.4 by the method of Ruffo *et al.* (9).

RESULTS AND DISCUSSION

When rats were fasted, the ATP-dependent CO_2-fixing activity of their liver cytosol was markedly reduced (60.8 $\pm$ 32.0 nmoles/min/g wet weight liver) compared with that of fed rats (132.5 $\pm$ 51.8 nmoles). Refeeding rats starved for 72 h resulted in a 3-fold increase in carboxylating activity to 205 $\pm$ 52.7 nmoles. These responses parallel those described for fatty acid synthesis (10) and the "oxoglutarate shunt" (1).

As previously described (11), the level of isocitrate dehydrogenase ($NADP^+$) did not vary significantly when the rats were subjected to the different dietary regimes.

Partial purification of the isocitrate synthase system, - The enzyme system responsible for the ATP-dependent reductive carboxylation of 2-oxoglutarate was partially purified as follows: livers from rats fasted and refed a high protein diet were homogenised in 3 volumes of 0.25 M sucrose containing 0.02 M N-ethylmorpholine, pH 7.5 and 0.001 M EDTA using a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 38,000 g for 30 min and the supernatant, which contained 80-85% of the synthase activity was freeze-dried and stored dessicated at -15°C. Isocitrate synthase activity was extracted from the lyophilised material using 2.5 volumes of a solution containing 20 mM N-ethylmorpholine, pH 7.5, 2.4 mM EDTA, 4.2 mM ATP and 8.4 mM $MgCl_2$. The solution was centrifuged at 38,000 g for 30 min and the pH of the supernatant adjusted to pH 6.0 with 1 M acetic acid. To this solution was added 0.1 volume of 5% (w/v) of streptomycin sulphate solution and the precipitate removed by centrifuging at 38,000 g for 15 min.

Finely ground polyethylene glycol (molecular weight, 20,000) was added to the supernatant to achieve a 4% (w/v) solution which was stirred for 30 min then centrifuged at 38,000 g for 20 min. To the supernatant was added more polyethylene glycol to raise the concentration to 12% (w/v), whereupon the precipitated protein was collected by centrifuging and redissolved in a minimum volume of buffer. The pH of the solution was adjusted to pH 5.5 with 1 M acetic acid and after 10 min, the precipitate was removed by centrifuging and discarded, while the pH of the supernatant was adjusted to

TABLE I Partial Purification of the Isocitrate Synthase System

Fraction	Specific activity	% Yield	Ratio of synthase to dehydrogenase+ activity
	(nmoles $^{14}CO_2$-fixed /min/mg of protein)		
Crude supernatant	4.9	100	0.009
Streptomycin sulphate supernatant	6.5	90	0.013
4-12% polyethylene glycol precipitate	13.8 *	72	0.028
pH5.5 supernatant	25.8 *	63	
20% acetone supernatant	39.4 *	49	0.066

* Values obtained when the enzyme solution had been incubated with ATP and Mg^{2+} for 60 min at 25°C prior to assay.

+ Isocitrate dehydrogenase activity measured in the forward direction.

pH 6.5. Freshly distilled acetone was added to a final concentration of 20% and again the precipitated protein was removed by centrifuging.

The yield and specific activity at each step in the purification procedure is presented in Table I.

Requirements of the isocitrate synthase system, - The data presented in Table II satisfy the requirements of equations (i) and (ii).

Identification of the products of the reaction, - When NADPH was included in the reaction mixture, all of the radioactivity in the acid stable products could be recovered in material identifiable as isocitrate by its co-chromatography both on thin layer

TABLE II. Requirements for the isocitrate synthase reaction.

	Enzymic activity (nmoles $^{14}CO_2$ fixed/min/mg of protein
Complete system	27.03
minus ATP	0.07
minus Mg^{2+}	0.02
minus NADPH	3.12
minus 2-oxoglutarate	0.85

cellulose plates and Whatman N0.3 paper with an authentic marker in the following solvent systems; ether/formic acid/water (10:2:15) adjusted to pH 1.7, R_f = 0.64; ethanol/ ammonia/water (80:5:15) adjusted to pH 6.5 with acetic acid, R_f = 0.64. However, when NADPH was omitted from the reaction mixture, all of the radioactivity due to $^{14}CO_2$-fixation was recovered in material which (a) co-chromatographed on Whatman No.3 paper using 5% $NaHCO_3$ as developing solvent, R_f = 0.89, and (b) co-electrophoresed at pH 8.8 in 0.02 M $(NH_4)HCO_3$ at 18 V/cm for 1 h (12), with the 2,4-dinitrophenylhydrazone of authentic oxalosuccinate.

To establish that the ATP-dependence of isocitrate synthase activity was a consequence of a stoichiometric relationship between $^{14}CO_2$-fixation and a 2-oxoglutarate-dependent hydrolysis of ATP, reaction mixtures were incubated with the enzyme system and either $H^{14}CO_3^-$ (0.25 μCi/μmole) or [γ-^{32}P]ATP (7.1 μCi/μmole). The orthophosphate released (27.4 $\pm$ 7.3 mμmoles) was determined after its separation from the [γ-^{32}P]ATP (13), and compared favourably with the 23.8 $\pm$ 2.1 mμmoles of $^{14}CO_2$ fixed.

The various pieces of evidence which clearly demonstrate that the reactions described in equations (i) and (ii) are catalysed by proteins distinct from isocitrate dehydrogenase are: (a) the isocitrate synthase system has an absolute requirement for ATP, (b) the increase in the ratio of isocitrate synthase to isocitrate dehydrogenase activity (measured in the forward direction) at successive steps in the purification procedure, (c) the greater sensitivity of the synthase activity to mild heat inactivation, (d) its response to dietary manipulation, and (e) the inhibition by 80 μM palmitoyl CoA of the synthase activity by 42% whilst having no effect on the activity of isocitrate dehydrogenase at this concentration.

Thus it appears that this hitherto undescribed enzyme system may fulfil the role previously ascribed to isocitrate dehydrogenase ($NADP^+$) of catalysing the conversion of 2-oxoglutarate to isocitrate in the synthesis of fatty acids from glutamate via the "oxoglutarate shunt".

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