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Effect of starvation on the activity of the mitochondrial tricarboxylate carrier

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Abstract

The effect of starvation on the activity of the tricarboxylate carrier has been investigated in intact rat liver mitochondria and in a reconstituted system. In both experimental conditions, the rate of citrate transport, when compared to control, is greatly reduced (35–40%) in starved rats. Similar behaviour is shown by the cytosolic lipogenic enzymes. Kinetic analysis of the carrier activity in intact mitochondria and in the proteoliposomal system has showed that during starvation only the V_{max} of this process decreases while there is no change in the K_m. No difference in the Arrhenius plot and in the lipid composition has been detected, which indicates that the reduced transport activity in fasted animals is not due to a change in the carrier lipid microenvironment. In starved rats, a reduction of the carrier activity has occurred even after the addition of increasing cardiolipin concentrations to proteoliposomes. These findings thus suggest that starvation-induced decrease of citrate carrier activity could be due to a change of the intrinsic properties of the transport protein.

Keywords: Starvation; Tricarboxylate carrier; Lipogenesis; Reconstitution; Mitochondrion; (Rat liver)

1. Introduction

The tricarboxylate carrier catalyzes an electroneutral exchange across the inner mitochondrial membrane of tricarboxylates, dicarboxylates and phospho*enol*pyruvate (for review see [1]). This carrier protein has been recently purified from rat and bovine mitochondria [2–4] and its primary structure has also been determined assigning this protein to the mitochondrial carrier family [5].

Beside other functions, the tricarboxylate carrier plays an important role in fatty acid biosynthesis because it is responsible for the transfer of acetyl-CoA, condensed with oxaloacetate in the form of citrate, from the mitochondria to the cytosol of the cell. Here, acetyl-CoA represents the primer for the de novo fatty acid biosynthesis. A great number of studies have indicated the enzymatic activities of this important metabolic process, i.e., acetyl-CoA carboxylase and fatty acid synthetase, are regulated by different nutritional and hormonal states (for review see [6]). The regulation of lipogenesis implies that the activity of the tricarboxylate carrier can be modulated in parallel. So far, little is known about the metabolic factors influencing the citrate carrier activity. In the past, a role of the mitochondrial citrate transporter was suggested in the regulation of fatty acid synthesis [7,8]. More recently, a modified transport activity of the tricarboxylate carrier has been found in mitochondria isolated from rats showing an insulin-dependent diabetes mellitus [9,10].

In this study, we have investigated the activity of the tricarboxylate carrier in mitochondria isolated from control and starved rats. Our studies have been performed on intact mitochondria and, at a second stage, on a reconstituted system in order to minimize the factors that can affect the transport activity in mitochondria. To our knowledge, this paper presents the first data on the effect of a nutritional state on the transport activity of liver tricarboxylate carrier functionally reconstituted in a liposomal system.

We have found that the citrate carrier activity is significantly reduced in starved rats. A coordination between the activities of lipogenic enzymes in the cytosol and citrate transport across the inner mitochondrial membrane has been found. Possible factors involved in the modulation of the tricarboxylate transport activity have also been investigated.

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2. Materials and methods

2.1. Materials

Hydroxyapatite (Bio-Gel HTP) and Bio-Rad Protein Assay were purchased from Bio-Rad; Amberlite XAD-2, Dowex AG1-X8, Pipes, Triton X-100, Triton X-114 and cardiolipin from Sigma; [1,5-^{14}C]citrate, [^{14}C]$NaHCO_3$ and [2-^{14}C]malonyl-CoA from Amersham International (Amersham, UK); egg yolk phospholipids (phosphatidylcholine from eggs) from Fluka; and Sephadex G-75 from Pharmacia. All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats (150–200 g), housed at a temperature of 22 ± 1°C, were used throughout these studies. They were fed ad libitum with a basal diet consisting of 25% protein, 4.3% lipid, 59.7% carbohydrate (of which 7.1% cellulose) and a salt and vitamin mixture. A second group of animals was starved for 48 h. Coprophagy was properly prevented.

2.3. Transport measurements in isolated rat liver mitochondria

Rat liver mitochondria were obtained as previously described [11]. Freshly isolated rat liver mitochondria were then loaded with malate essentially as described [12]. To this purpose they were incubated (about 40–50 mg protein) at 20°C in 10 ml of 100 mM KCl, 20 mM Hepes, 1 mM EGTA, pH 7.0, in the presence of 2 μg/ml rotenone and 0.75 mM L-malate. After 2 min, 20–30 μmol/g protein of mersalyl was added in order to inhibit the dicarboxylate carrier. After 1 min at 20°C, the mitochondria were diluted with the above reported ice-cold buffer and centrifuged at 20000 × *g* for 5 min at 2°C. The reisolated mitochondria were then washed once and finally resuspended in 1.5 ml of the same buffer. The kinetics of [^{14}C]citrate/malate exchange were determined by using the inhibitor stop method according to [12]. Malate-loaded mitochondria (1–2 mg of protein) were incubated in 1 ml of reaction mixture containing 100 mM KCl, 20 mM Hepes, 1 mM EGTA, pH 7.0 and 2 μg rotenone. The temperature, unless otherwise specified, was 9°C. The exchange was started by adding [^{14}C]citrate and terminated, after the indicated times, by the addition of 12.5 mM 1,2,3-benzenetricarboxylate (1,2,3-BTA). The mitochondria were then immediately reisolated by centrifugation, washed once and acidified with 20% $HClO_4$. The supernatant obtained after centrifugation was diluted into 7 ml of scintillation mixture, vortexed and counted.

2.4. Isolation and reconstitution of the tricarboxylate carrier

The tricarboxylate carrier was isolated and reconstituted in liposomes, as described [2], with some modifications. Rat liver mitochondria were extracted with 3% Triton X-100 (w/v), 20 mM Na_2SO_4, 1 mM EDTA and 10 mM Pipes (pH 7.0) at a final concentration of 10–12 mg protein/ml. After 10 min at 0°C the extract was centrifuged at 25000 × *g* for 20 min at 2°C. 600 μl of supernatant were applied to cold hydroxyapatite columns and eluted with a buffer containing 0.5% Triton X-100 and 5 mM citrate (pH 7.0). The first 0.6 ml of hydroxyapatite eluate were collected. Liposomes were prepared by sonication of egg yolk phospholipids in water (100 mg/ml). The composition of the reconstitution mixture was: 20 μl of mitochondrial extract or 50 μl of hydroxyapatite eluate, 90 μl of 10% Triton X-114, 20 μl of 20 mg/ml cardiolipin, 100 μl of 10% phospholipids in the form of sonicated liposomes, 70 μl of 100 mM Pipes (pH 7.0) and 35 μl of 200 mM citrate in a final volume of 700 μl. This mixture was then passed 15 times through the same Amberlite XAD-2 column (0.5 × 3.6 cm) preequilibrated with a buffer containing 10 mM Pipes and 10 mM citrate (pH 7.0). All the above described operations were performed at 4°C, except for the passage through Amberlite, which was carried out at room temperature.

2.5. Transport measurements in proteoliposomes

For transport measurements the procedure, already described by Bisaccia et al. [2], was essentially followed. Citrate present outside the proteoliposomes was removed by passing them through a Sephadex G-75 column preequilibrated with 50 mM NaCl and 10 mM Pipes (pH 7.0). The turbid eluate collected from the Sephadex column was transferred to reaction vessels (180 μl each). After incubation for 3 min at 25°C, transport measurements were started by adding 0.5 mM [^{14}C]citrate (about 100000 cpm) to proteoliposomes. Transport was stopped, after the desired time interval, by the addition of 20 mM 1,2,3-BTA. The same amount of 1,2,3-BTA was added together with the labelled substrate at time zero in control samples. The radioactivity external to proteoliposomes was removed by passing them through Dowex AG1-X8 columns, 100–200 mesh, chloride form (0.5 × 4 cm, equilibrated with 50 mM NaCl). The samples were eluted with 1.2 ml of 50 mM NaCl, collected in 4 ml of scintillation mixture, vortexed and counted.

2.6. Assay of lipogenic enzymes

Acetyl-CoA carboxylase and fatty acid synthetase activities were assayed in rat liver cytosol. This was obtained by centrifuging the post-mitochondrial supernatant at 20000 × *g* for 20 min. The pellet was discarded and the supernatant was then centrifuged at 105000 × *g* for 1 h. On the resulting cytosol the assay for acetyl-CoA carboxylase quantified [^{14}C]$NaHCO_3$ fixation into an acid-stable product during a 3-min reaction period [13]. The activity of fatty acid synthetase was assayed by following the incor-

poration of [2-^{14}C]malonyl-CoA into fatty acids. At the end of the incubation period, the reaction was stopped by ethanolic KOH. After saponification for 1 h, fatty acids were extracted with petroleum ether and their radioactivity counted as in [14].

2.7. Other methods

Protein was determined by the method of Lowry et al., modified for the presence of Triton [15], and by the method of Bradford [16].

3. Results

3.1. De novo fatty acid synthesis enzymatic activities

Table 1 reports the effect of starvation on the activities of the lipogenic enzymes. The activity of acetyl-CoA carboxylase, the first committing step in fatty acid biosynthesis, was reduced by about 60% in starved rats (0.27 ± 0.02 vs. 0.67 ± 0.04 nmol/min per mg protein of the control). Fatty acid synthetase activity decreased to the same extent as carboxylase activity during starvation.

3.2. Characterization of citrate transport activity in intact liver mitochondria

The measurements of the transport activity of the tricarboxylate carrier were performed in freshly isolated mitochondria by using the inhibitor stop method as previously reported [12]. The time-course of the citrate uptake at 9°C by malate-loaded mitochondria isolated from control and starved rats is shown in Fig. 1. In all experiments, the incubation mixture contained equal amounts of mitochondrial proteins and an external citrate concentration of 0.5 mM. The activity of the tricarboxylate carrier in mitochondria from 48 h starved rats was significantly lower than that obtained in mitochondria from normally fed rats. Citrate uptake increased linearly with time up to 20 s. At this time, the carrier activity in liver mitochondria isolated from starved rats was reduced by about 40%. Results obtained from twelve different experiments are the following: 2.4 ± 0.18 vs. 3.9 ± 0.32 nmol/mg protein of the control. In other experiments (not shown) the decrease of the tricarboxylate transport activity was monitored in starved rats in relationship to the fasting time of the animals. A clear decrease of the transport activity was already evident after 12 h fasting reaching the lowest level (about 35–40% drop) in mitochondria isolated from 48 h starved rats. Therefore, this starvation period has been chosen in subsequent experiments.

Table 1
Effect of starvation on the activities of acetyl-CoA carboxylase and of fatty acid synthetase in rat liver cytosol

Animals	Acetyl-CoA carboxylase (nmol [^{14}C]$NaHCO_3$ incorporated per min per mg protein)	Fatty acid synthetase (nmol [2-^{14}C]malonyl-CoA incorporated per min per mg protein)
Control	0.67 ± 0.04	0.83 ± 0.09
Starved	0.27 ± 0.02	0.36 ± 0.06

Data are the means ± S.D. of five experiments, with each determination carried out in triplicate.

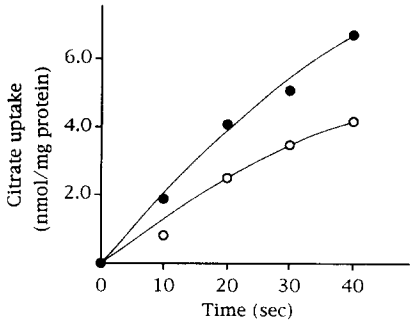

Fig. 1. Time-course of citrate uptake by liver mitochondria isolated from control and starved rats. Rat liver mitochondria (2 mg protein) loaded with malate, as described in Section 2, were incubated for 3 min in 1 ml of reaction mixture containing 100 mM KCl, 20 mM Hepes, 1 mM EGTA, pH 7.0 and 2 μg rotenone at 9°C. The exchange reaction was started by adding 0.5 mM [^{14}C]citrate and terminated, after the indicated times, by the addition of 12.5 mM 1,2,3-BTA. The mitochondria were then reisolated by centrifugation. Other details are reported in Section 2. ●, Mitochondria from control rats; ○, mitochondria from starved rats.

Thereafter the kinetic parameters of the tricarboxylate carrier by mitochondria from control and starved rats have been determined by studying the dependence of the citrate/malate exchange rate on the different substrate con-

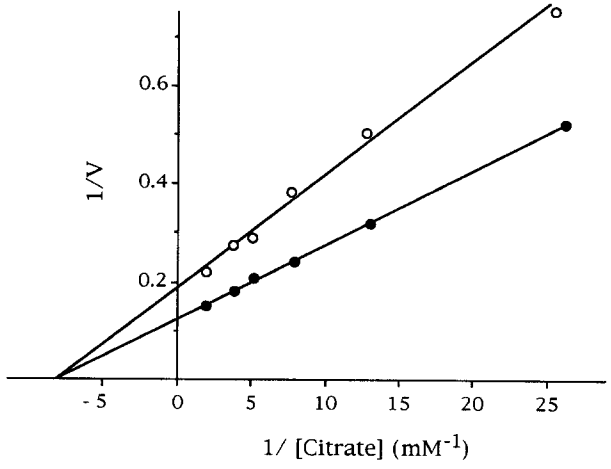

Fig. 2. Dependence of the rate of citrate uptake on substrate concentration in liver mitochondria from control and starved rats. Malate-loaded mitochondria (2 mg protein) were incubated under the conditions described in the legend of Fig. 1, except that [^{14}C]citrate was added at the concentrations indicated. The temperature was 4°C. The citrate/malate exchange was stopped 15 s after the addition of [^{14}C]citrate by 12.5 mM 1,2,3-BTA. V is expressed as nmol per min per mg protein. ●, Mitochondria from control rats; ○, mitochondria from starved rats.

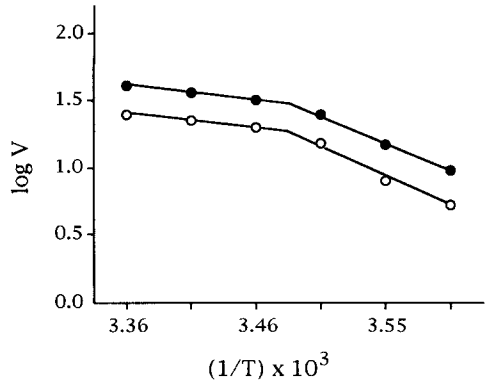

Fig. 3. Arrhenius plot of the temperature dependence of the rate of citrate uptake in liver mitochondria isolated from control and starved rats. Malate-loaded mitochondria (2 mg protein) were incubated as described in the legend of Fig. 1, except that the temperature was varied as indicated. V is expressed in nmol per min per mg protein. ●, Mitochondria from control rats; ○, mitochondria from starved rats.

centrations (externally added to liver mitochondria). Fig. 2 shows that in mitochondria isolated from starved rats V_{max} was the only kinetic parameter of citrate transport to be significantly influenced. V_{max} decreased by about 35% while no significant change of K_m was recorded. The following results have been obtained in five different experiments: control rats, K_m 0.133 ± 0.009 mM and V_{max} 8.6 ± 0.5 nmol/min per mg protein, values which are in good agreement with previous data from other laboratories [12,17]; starved rats, K_m 0.138 ± 0.014 mM and V_{max} 5.9 ± 0.6 nmol/min per mg protein. These results indicate that during starvation the affinity of the tricarboxylate carrier for its substrate is essentially the same.

Fig. 3 shows the temperature dependence of the citrate/malate exchange in liver mitochondria from both control and fasted rats. The Arrhenius plot resulted to be biphasic with two lines intersecting at about 14°C for both kinds of mitochondrial preparations. This indicates that membrane fluidity is similar in control and starved rats. Further information on the mitochondrial membrane phospholipid and fatty acid compositions, which strongly influence membrane fluidity [18,19], have been obtained by HPLC and gas-liquid chromatography analysis, respectively. No significant variation was detected in both phospholipid and fatty acid compositions of mitochondrial membranes isolated from starved rats as compared to control (data not shown).

3.3. Properties of the reconstituted citrate transport activity

In order to avoid all the complications involved in citrate transport assay with intact mitochondria [9], a second experimental approach has been used consisting in the reconstitution of the Triton X-100 mitochondrial extract and the hydroxyapatite eluate into liposomes. In both cases it has been found that the level of functional tricarboxylate carrier obtained from starved rat mitochondria was reduced by about 40% as compared to that of control rats (data not shown).

The dependence of the exchange rate on substrate concentration has then been studied in proteoliposomes reconstituted with hydroxyapatite eluates derived from control and starved rat mitochondria. Fig. 4 shows the results of a typical experiment as Lineweaver-Burk plot. V_{max} value resulted to be significantly lower for the reconstituted tricarboxylate carrier isolated from starved rat mitochondria. On the contrary, identical K_m values for both kinds of proteoliposomes were observed. These results are similar to those previously obtained with intact mitochondria (see Fig. 2) and corroborate the finding that the affinity of the tricarboxylate carrier for its substrate is unchanged during starvation.

The influence of different phospholipids on the reconstituted tricarboxylate transport activity has been previously investigated [20–22] and it has been found to be stimulated by the presence of cardiolipin during reconstitution. Therefore we have investigated the effect of cardiolipin on the hydroxyapatite eluate reconstituted into liposomes. Fig. 5 shows that the specific activity of the reconstituted tricarboxylate carrier from control rats grew on increasing the concentration of cardiolipin present in the liposomal membranes. Maximal activation was reached at 4% (w/w) of cardiolipin. The specific activity of the tricarboxylate carrier from starved rats showed the same characteristic activation by cardiolipin. Noteworthy is that the decrease of the carrier activity in starved rats was not reversed by increasing the concentration of cardiolipin. This means that variations of the cardiolipin amount in mitochondrial membranes are not the factor responsible for the decreased

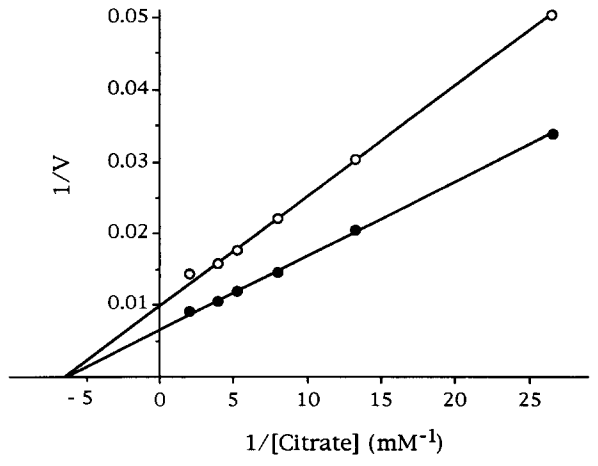

Fig. 4. Dependence of the rate of citrate uptake on substrate concentration in proteoliposomes. Proteoliposomes were prepared with hydroxyapatite eluates from control (●) and starved rats (○). [^{14}C]citrate was added, at the concentrations indicated, to proteoliposomes containing 10 mM citrate as internal substrate. The exchange reaction was stopped 1 min after the addition of [^{14}C]citrate by the addition of 20 mM 1,2,3-BTA. V is expressed as nmol per min per mg protein. Other details are reported in Section 2.

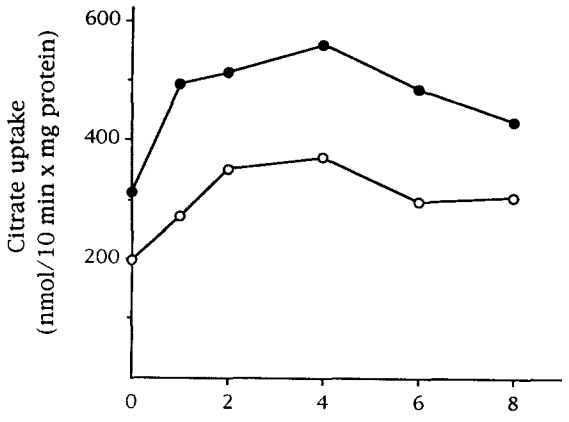

Fig. 5. Dependence of the citrate uptake on the concentration of cardiolipin added to proteoliposomes. Proteoliposomes were prepared with hydroxyapatite eluates from control (●) and starved rats (○). The concentration of cardiolipin with respect to egg yolk phospholipids was varied as indicated. The exchange reaction was started by adding 0.5 mM [^{14}C]citrate to proteoliposomes containing 10 mM citrate as internal substrate. Other details are reported in Section 2.

tricarboxylate transport activity found in rats during starvation.

4. Discussion

The citrate carrier plays an important role in the transport of acetyl-CoA from the mitochondria to the cytosol, where hepatic lipogenesis occurs. To date, little attention has been paid to factors regulating the carrier activity [8,9]. The results presented in this paper demonstrate that the activity of the tricarboxylate carrier depends on the nutritional state of the animal, indeed it is strongly reduced during starvation. The carrier activity parallels that of the cytosolic lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthetase, which strongly decreases in starved rats. The kinetic analysis (Fig. 2) of the citrate carrier activity we have obtained indicates that only the V_{max} of the citrate uptake is decreased during starvation, while there is no change in the affinity of the protein for its substrate in mitochondria isolated from both normally fed and starved rats ($K_m = 0.133 \pm 0.009$ mM and 0.138 ± 0.014 mM, respectively). These results are in contrast with those reported by Halperin et al. [7,8], who found an unchanged V_{max} and increased K_m values for the citrate carrier activity in mitochondria from starved rats. Such a discrepancy is difficult to explain. Note that in the quoted studies the authors determine the citrate transport activity in liver mitochondria by studying the internal [^{14}C]citrate/external citrate exchange. Under these conditions, not only [^{14}C]citrate, but also labelled isocitrate and *cis*-aconitate, formed inside the mitochondria, are transported [23,24]. Moreover, a series of reactions are needed to obtain mitochondrial [^{14}C]citrate loading [23]. In the present study the citrate transport activity has been determined following the external [^{14}C]citrate/internal malate exchange, which, as shown by Palmieri et al. [12], represents a simpler and more efficient way of investigation. The V_{max} values we found with this method, in agreement with other laboratories [12,17], are several times higher than those reported by Halperin et al. [7,8]. The lower affinity of the carrier for citrate was ascribed by the latter authors to the starvation-induced increase in long-chain fatty acyl-CoA and in fatty acid oxidation [7,8]. However, other authors have argued that inhibition of citrate transport by long-chain acyl-CoA esters is of questionable physiologic significance [25]. To clarify this discrepancy of results, we have further investigated this problem by using the experimental approach of the functional reconstitution in liposomes of the tricarboxylate carrier, deriving from both starved and normally fed rats. The reconstitution of carrier proteins in liposomes has been demonstrated to have several advantages [9,10], among others the carrier assay in a liposomal system reflects the intrinsic properties of a given transport protein rather than the altered substrate and/or effector levels, which may exist in starved versus control animals. The kinetic analysis of the tricarboxylate carrier activity reconstituted into the proteoliposomal system reported in Fig. 4, shows the same behaviour as obtained with intact mitochondria (Fig. 2), i.e., a practically unchanged K_m and a significant decrease of V_{max} value in starved animals. Under the experimental conditions we used, the hydroxyapatite eluate is diluted about 15-times in the reconstitution mixture. Nevertheless, a reduced carrier activity, similar to that found in intact mitochondria, is observed. This makes it unlikely that palmitoyl-CoA represents a real modulator of citrate carrier as suggested by Halperin et al. [7,8,26]. It is worth underlining that an unchanged K_m value for the citrate carrier activity has been recently reported in mitochondria from hyperthyroid rats [17], in which the increased level of fatty acyl-CoA and of fatty acid oxidation are well known to occur [27]. To our knowledge, this is the first report on the effect of a nutritional state on the activity of the tricarboxylate carrier solubilized and reconstituted in a liposomal system.

It is well known that membrane fluidity can affect the activities of many membrane associated enzymes [18,19], among others that of the tricarboxylate carrier [17]. However, the temperature dependence of citrate transport in starved rats resulted to be comparable with that of control rats (Fig. 3). This could indicate that no detectable change in mitochondrial membrane fluidity and then in the carrier lipid microenvironment has occurred during starvation. Moreover, in starved animals, the same decrease of transport activity has been shown by the tricarboxylate carrier during reconstitution in artificial membranes formed mainly by phosphatidylcholine and cardiolipin. It has been shown that altered thyroid hormone levels can influence the citrate carrier activity [17] as well as the activity of other mitochondrial proteins [28,29] by changing the cardiolipin

content of the mitochondrial membrane. Recently, Klingenberg et al. [30] have indicated that a deficiency in cardiolipin binding by an ADP/ATP carrier mutant is reversed by the addition of cardiolipin on reconstitution. Our results in Fig. 5, however, indicate that the decrease of the tricarboxylate transport activity observed during starvation is not reversed by increasing the amount of cardiolipin in the reconstituted system.

Lastly, it must be considered that citrate, in the cell cytoplasm, serves as substrate in ATP-citrate lyase reaction as well as an activator of acetyl-CoA carboxylase [6]. Then the inhibition of lipogenesis during starvation could be explained at least in part by a reduction of the cytosolic citrate level, due in its turn to a decrease in the citrate carrier activity.

Taken together, our results indicate that the reduced tricarboxylate transport activity in starved rats could depend from a change of the intrinsic properties of the carrier protein rather than from other factors that may vary in mitochondria during starvation. However, a starvation induced reduction of the amount of the carrier protein cannot be excluded. We are currently performing experiments in order to gather more information at the molecular level on the starvation-induced decrease of the citrate carrier activity.

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