CONTROL OF CITRATE FORMATION IN RAT LIVER MITOCHONDRIA *

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Received May 10, 1967

The importance of citrate as a regulatory metabolite of cellular enzyme systems has been discussed recently by Srere (1965). In addition to its role as an intermediate of the Krebs cycle, postulated extra-mitochondrial functions are: a source of reducing power and acetyl CoA in the cystosol, and a regulator of the rate-limiting enzymes of fatty acid synthesis and glycolysis, acetyl CoA carboxylase and phosphofructokinase, respectively. Control of citrate formation acquires added significance in liver because the condensation of acetyl CoA with oxalacetate to form citrate is rendered non-obligatory by the ability of liver to oxidize fatty acids to ketones (Löffler, Matchinsky and Wieland, 1965).

Shepherd and Garland (1966) have recently confirmed inhibition of citrate synthase by ATP and other nucleotides with enzyme from rat liver, which was first observed with yeast citrate synthase by Hathaway and Atkinson (1965). In further studies, Shepherd, Yates and Garland (1965) suggested that palmitoyl carnitine in the presence of malate was oxidized primarily to acetoacetate with rat liver mitochondria in the presence of ADP, and to citrate if oxidative phosphorylation was uncoupled. They proposed that increased intramitochondrial ATP synthesis, associated with the oxidation of fatty acids to acetyl CoA, provided a switch for the diversion of acetyl CoA from citrate towards acetoacetate synthesis when fatty acid availability was increased.

The ratio of ATP/ADP in total tissue extract, however, has been shown to decrease, both upon addition of fatty acids to perfused rat liver (Williamson, Kreisberg and Felts, 1966), and in livers from ketotic rats (Wieland, 1965). Furthermore, Kosiki and Lee (1966) have demonstrated that the nucleotide inhibition of citrate synthase may be reversed by 5 to 10 mM Mg^{++}.

*Supported by grants from the PHS (12202-04) and American Heart Assoc.

In view of a possible uncertainty about the physiological significance of ATP control at the citrate synthase site, the problem has been reinvestigated in rat liver mitochondrial preparations respiring without addition of external nucleotides.

Methods. Rat liver mitochondria with respiratory control ratios of 5 to 7 in the presence of succinate and rotenone were prepared according to Chance and Mela (1966), with the exception that 50 μM EGTA was used instead of EDTA in the isolation procedure. Mitochondrial incubations were conducted at 28 ° in a sampling device which permits continuous oxygen tension recordings with rapid sample ejection into perchloric acid at desired intervals (final conc. 6 °/o w/v). The basic incubation medium consisted of 246 mM sucrose, 2 mM KCl, 20 mM Tris-Cl and 5 mM Tris-phosphate, pH 7.2. Other additions were as follows: 5 mg/ml bovine serum albumin, fraction V (Sigma Chemical Co.), defatted by charcoal treatment (Chen, 1967); 10 μM fluorocitrate (generously provided by Dr. E. Kun); 250 μM Tris malate; 5 mM glucose; 200 μg/ml hexokinase , Type V (Sigma Chemical Co.), and 50 μM (-) palmitoyl carnitine (generously provided by Dr. I. B. Fritz). Oligomycin was purchased from Sigma Chemical Co. and atractyloside was a gift from Dr. H. Rasmussen. Metabolic intermediates were measured in neutralized perchloric acid extracts of mitochondria by fluorometric enzyme methods (Williamson, 1965). ATP was measured by the glyceraldehyde-3-P-dehydrogenase:P-glycerate kinase coupled enzyme system (Bergmeyer, 1963). Citrate was determined with citrate lyase (Moellering and Gruber, 1966), and protein by the biuret method (Layne, 1957).

Results and Discussion. Inhibition of aconitase by fluorocitrate (Peters, Wakelin and Buffa, 1953) allowed citrate accumulation to be used as a monitor of flux through the citrate synthase step. Respiration of mitochondria with 2 mM K^+ in the incubation medium was initiated by the addition of hexokinase after a 2 min preincubation period. Rates of 30 mμ atoms O_2/mg/min were obtained without addition of Mg^{++} or ADP to the mitochondrial incubation. Fig. 1A shows that the respiratory rate was stimulated 36 °/o by 0.6 μM p-trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP) as an uncoupling agent, and was inhibited 74 °/o by oligomycin and 58 °/o by atractyloside. Fig. 1B shows that the rate of citrate formation was doubled by FCCP and inhibited 90 °/o by oligomycin and 83 °/o by atractyloside. These results show that citrate formation was stimulated more than respiration by FCCP and inhibited more than respiration by either oligomycin or atractyloside.

ATP accounted for about 50 °/o of the total endogenous adenine nucleotides during glucose-hexokinase stimulated respiration (Fig. 2). Oligomycin (see Lee and Ernster, 1966) caused a rapid increase of ADP at the expense of ATP. Atractyloside and FCCP (0.6 μM) decreased ATP levels by similar

amounts, accounted for primarily by an increase of ADP with FCCP and of AMP with atractyloside (Fig. 2B and C). Atractyloside has been shown to inhibit

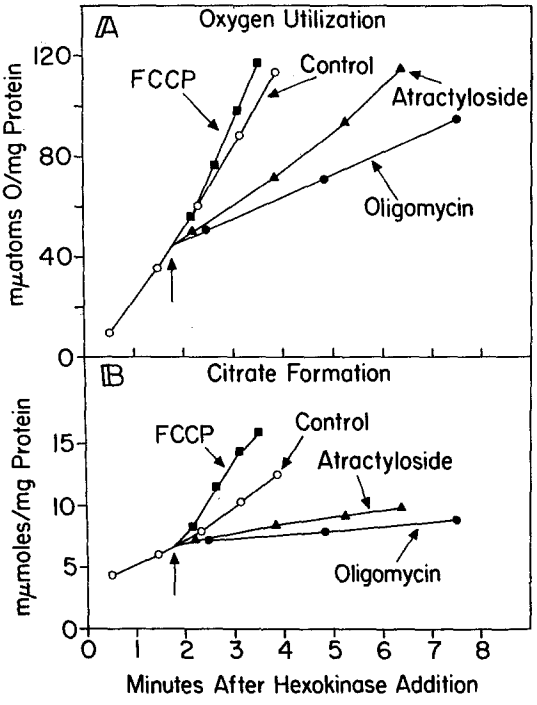

Fig. 1. Oxygen utilization and citrate production by rat liver mitochondria (2.8 protein mg/ml) oxidizing (-) palmitoyl carnitine and malate. Atractyloside (7.4 μM), oligomycin (2.35 μg/ml), or FCCP (0.6 μM) were added at the time denoted by the arrow. Hexokinase was added at zero time after a 2 min incubation period.

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the translocation of ATP and ADP through a barrier situated between the extramitochondrial phase and the site of oxidative phosphorylation (Chappell and Crofts, 1965; Klingenberg and Pfaff, 1966). The increased displacement from equilibrium of the adenylate kinase mass action ratio in the presence of atractyloside (Fig. 2D) provides direct evidence for the location of adenylate kinase outside the atractylate-sensitive barrier (Klingenberg and Pfaff, 1966).

The results in Figs. 1 and 2 clearly demonstrate a lack of correlation between the endogenous ATP content of liver mitochondria and the rate of conversion of palmitoyl carnitine to citrate in the presence of malate. Thus, FCCP promoted citrate formation with a small decrease of ATP, while atractylate and oligomycin greatly inhibited citrate formation (along with respiration) with either a small decrease of ATP (atractylate) or a large decrease (oligomycin).

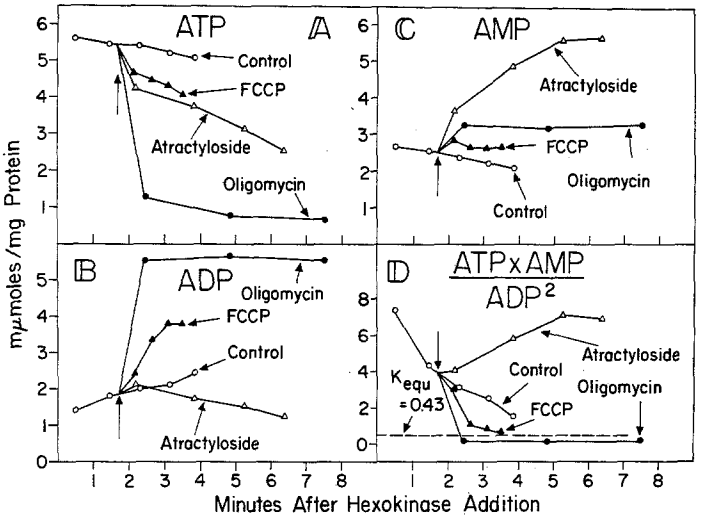

Fig. 2. Changes of endogenous adenine nucleotides after the addition of 7.4 μM atractyloside, 2.35 ug/ml oligomycin or 0.6 μM FCCP to rat liver mitochondria (2.8 mg protein/ml), oxidizing (-) palmitoyl carnitine and malate. Kequ refers to the equilibrium constant of adenylate kinase.

The explanation for the effects of these agents on the rate of citrate accumulation in the mitochondrial system resides in their relative effects on the redox state of the intramitochondrial pyridine nucleotides (Fig. 3). Addition of hexokinase to the incubation medium caused an initial rapid oxidation of pyridine nucleotides, followed by a slow drift to a more oxidized state. Atractyloside and oligomycin produced a reduction of pyridine nucleotides, denoted by a fluorescence increase. FCCP, however, caused a rapid shift of the pyridine nucleotides to the oxidized state. The intramitochondrial oxalacetate level is determined by the redox potential of the NAD^+ system reacting with malate dehydrogenase, because malate was added in excess, and the Krebs cycle blocked at aconitase. In accordance with Lehninger's proposal (Lehninger, 1946), we suggest that the conversion of acetyl CoA to either citrate or acetoacetate is determined primarily by mitochondrial oxalacetate availability, which is, itself, related directly to the redox state of mitochondrial pyridine nucleotides.

The poor correlation between endogenous mitochondrial ATP levels and the rate of citrate formation was confirmed in other experiments (Williamson and Olson, 1967) in which palmitoyl carnitine respiration was initiated by either valinomycin (Höfer and Pressman, 1966), or 2-methyl-1,4-naphthoquinone (vitamin K_3) in the presence of rotenone. Respiration and citrate formation were oligomycin insensitive, but ATP levels decreased to 1-2 mμmoles/mg protein in

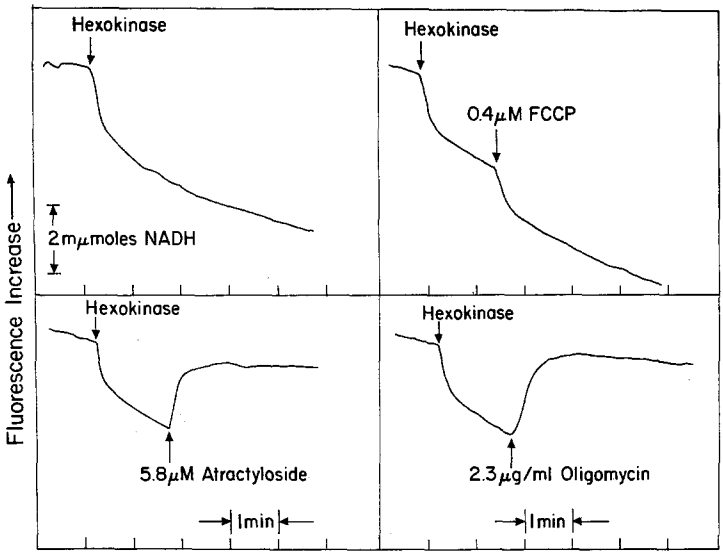

Fig. 3. Nicotinamide nucleotide fluorescence traces of rat liver mitochondria (2.6 mg protein/ml) oxidizing (-) palmitoyl carnitine and malate.

the presence of oligomycin. Furthermore, addition of 2 mM ATP plus 5 mM $MgCl_2$ to the oligomycin-inhibited systems failed to affect the rate of citrate synthesis (cf. Shepherd, et al., 1965).

Inhibition of citrate synthase by fatty acyl CoA described by Wieland, Weiss and Eger-Neufeldt (1964) is obviated in the present experiments, since (-) palmitoyl carnitine has been shown by Fritz (1966) to prevent palmitoyl CoA inhibition of citrate synthase. Direct measurements of long-chain fatty acyl CoA derivatives in the deproteinized mitochondrial pellet supported this conclusion.

Summary. Measurements of citrate formation and endogenous adenine nucleotides in fluorocitrate-inhibited rat liver mitochondria respiring in the presence of 50 μM (-) palmitoyl carnitine and 250 μM malate have failed to support the suggestion that citrate synthase activity is controlled primarily by the intramitochondrial ATP level. A positive correlation was obtained between the redox state of the mitochondrial pyridine nucleotides and the rate of citrate formation, suggesting that at physiological malate concentrations, the rate of entry of acetyl CoA into the citric acid cycle is effectively controlled by oxalacetate availability.

As acetyl CoA levels increase, and oxalacetate levels decrease, in rat liver with increased fatty acid availability (Williamson, Herczeg, Coles and

Danish, 1966; Williamson, 1967), increased ketogenesis accompanies the observed increase in the redox state of pyridine nucleotides in both the cytoplasmic and mitochondrial spaces. We suggest that the change in the redox state following increased fatty acid oxidation is a primary event preceding ketosis.

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