INHIBITION OF CITRATE SYNTHASE BY SUCCINYL-CoA AND OTHER METABOLITES

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1. Introduction

Regulation of citric acid cycle flux through a direct allosteric inhibition of citrate synthase by ATP has been postulated by Hathaway and Atkinson [1] and by Shepherd and Garland [2]. Experimental support for this conclusion was provided by Garland et al. [3, 4] in studies with isolated rat liver mitochondria. However, doubts concerning a direct involvement of ATP in the physiological regulation of citrate synthase arose from the demonstration that inhibition by ATP in kinetic experiments could be reversed by Mg^{2+} [5], and from mitochondrial studies of other workers which supported an indirect regulation by ATP via the effect of the NADH/NAD ratio on oxalacetate availability [6–11]. More recent experiments, which examined control of the citric acid cycle in the absence of inhibitors, supported the existence of a feedback control between substrate-level phosphorylation at succinic thiokinase, effective only at high oxalacetate and low acetyl-CoA concentrations, and flux through citrate synthase [12, 13]. Under these conditions, citrate synthase flux showed an inverse correlation with the measured succinyl-CoA content of the mitochondria, raising the possibility of direct inhibition of citrate synthase by succinyl-CoA. The kinetic experiments reported here with purified citrate synthase from beef heart and rat liver show that succinyl-CoA is a much stronger inhibitor of citrate production than ATP, strictly competitive with acetyl-CoA and noncompetitive with respect to oxalacetate. Furthermore, carefully controlled rate measurements show no kinetic indication of cooperativity in the presence or absence of either ATP or succinyl-CoA. Citrate, propionyl-CoA and CoA are also specific inhibitors at concentrations of physiological significance.

2. Experimental procedures

Acetyl-CoA, CoA (chromatographically pure) and CoA derivatives were obtained from P-L Biochemicals, Inc. This acetyl-CoA was identical kinetically to that prepared from acetic anhydride and CoA after purification by DEAE-cellulose and Biogel P-2 chromatography as described by C. Fung and M.F. Utter (personal communication). The concentration was determined by assay with α-ketoglutarate dehydrogenase and phosphotransacetylase [14]. The succinyl-CoA was 90–92% pure, determined by assay with α-ketoacyl-CoA transferase and β-hydroxybutyryl-CoA dehydrogenase, and the absorbance at 258 nm. Succinyl-CoA, prepared from succinic anhydride and CoA [15] and chromatographed on DEAE-cellulose (formate form) according to Cha et al. [16], was used to verify the inhibition obtained with the commercial preparation. Propionyl-CoA was measured from the 258 nm absorbance using the specifications of P-L Biochemicals, Inc. The CoA content of the acyl-CoA derivatives, determined by assay with α-ketoglutarate dehydrogenase, was negligible.

Citrate synthase was purified from beef heart and crystallized to a specific activity of 162 I.U. by the procedure of Srere [17]. The same procedure, with slight modifications, was used to prepare the enzyme from rat liver mitochondria.

Table 1
Kinetic parameters for the rate of oxalacetate utilization by citrate synthase.

Enzyme source	Inhibitor	K_i (μM)	Type with respect to acetyl-CoA	Type with respect to oxalacetate
Beef heart	ATP	950	Mixed	Noncompetitive
Beef heart	CoA	67	Mixed	Noncompetitive
Beef heart	Succinyl-CoA	130	Competitive	Noncompetitive
Beef heart	Propionyl-CoA	50	Competitive	Noncompetitive
Beef heart	Citrate	1600	Noncompetitive	Competitive
Rat liver	Succinyl-CoA	140	Competitive	Noncompetitive

The K_m for acetyl-CoA with the beef heart and rat liver enzymes were 8 and 10 μM, respectively. The K_m for oxalacetate with the beef heart enzyme was 1.6 μM.

The rate of citrate production was followed on a modified Eppendorf fluorometer with a coupled assay system using NAD, malate and a large excess of malate dehydrogenase to produce equilibrium concentrations of oxalacetate and NADH [2]. Full-scale deflection was obtained with 0.5 μM NADH over the range of 1–20 μM NADH and initial velocities were measured using 20% of the scale. All reactions were performed in 0.1 M Tris-Cl, pH 7.4, 21° with 1 to 10×10^{-5} mM citrate synthase.

3. Results and discussion

The kinetics of citrate production were examined with crystalline citrate synthase from beef heart. Variation of one substrate at a series of fixed levels of cosubstrate gave apparent Michaelis constants for acetyl-CoA and oxalacetate independent of the concentration of cosubstrate. This finding is consistent with a mechanism of "rapid-equilibrium", random addition of substrates as reported by Shepherd and Garland for the rat liver enzyme [2], and by Moriyama and Srere [18] using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay system. Over the range of acetyl-CoA concentrations (2–100 μM) and oxalacetate levels (1–10 μM) examined there was no evidence of substrate inhibition. Kinetic patterns and parameters are summarized in table 1.

Care was taken to examine ATP inhibition at low concentrations of oxalacetate and under conditions where Shepherd and Garland [2] had observed an allosteric inhibition by ATP. The ATP concentration used did not contribute significantly to the ionic strength. In contrast to the work of Jangaard et al. [19] and Shepherd and Garland [2], ATP variation introduced no signs of sigmoidicity into kinetic plots, and the inhibition was noncompetitive with respect to oxalacetate at all oxalacetate concentrations examined (fig. 1). The absence of allosteric inhibition is consistent with physical studies of citrate synthase which report two identical subunits [18, 20]. Double reciprocal plots of acetyl-CoA varied at several fixed levels of ATP intersected on or slightly to the left of the ordinate, corresponding to about 50 μM acetyl-CoA. An identical kinetic pattern (mixed inhibition) and intersection point were obtained with CoA as an inhibitor (table 1). However, CoA inhibition has no parallel in intact rat heart mitochondria, where the directional changes of CoA and flux through citrate synthase are the same [12, 13]. Since CoA inhibition is not strictly competitive with respect to acetyl-CoA, its strength relative to that of the inhibition by acyl-CoA derivatives may vary greatly with the conditions

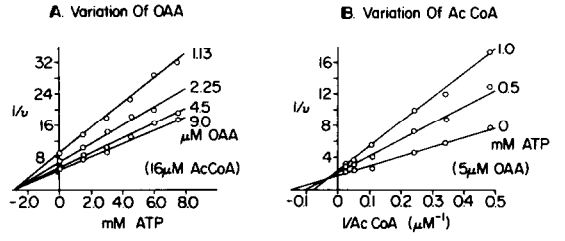

Fig. 1. Inhibition of beef heart citrate synthase by ATP in 0.1 M Tris-Cl, pH 7.4, 21°. Velocities are expressed in arbitrary units.

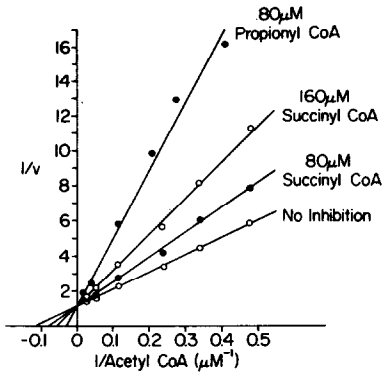

Fig. 2. Inhibition of citrate synthase by succinyl-CoA and propionyl-CoA in 0.1 M Tris-Cl, pH 7.4, 21°. Oxalacetate concentration, fixed by the malate dehydrogenase equilibrium, was 3.1 μM.

used for kinetic measurements. Preliminary experiments varying ionic strength tend to support this conclusion. Alternatively, all the measured intramitochondrial CoA may not be fully accessible to citrate synthase at any given time.

With the exception of succinyl-CoA, citrate is the strongest inhibitor among the citric acid cycle intermediates with a K_i of 1.6 mM. Citrate concentrations range at and above this level [13] and may provide an effective product inhibition at low oxalacetate concentrations. α-Ketoglutarate had a K_i greater than 10 mM and was not competitive with respect to either substrate.

Both succinyl-CoA and propionyl-CoA are competitive inhibitors with respect to acetyl-CoA and purely non-competitive with respect to oxalacetate (fig. 2). This is in marked contrast to the inhibition by palmitoyl-CoA, once postulated to be a regulator of citrate synthase [21, 22] and presently assumed to inhibit by a nonphysiological detergent-like action [23]. Palmitoyl-CoA inhibition was not competitive with acetyl-CoA, but depended on the oxalacetate concentration, the ratio of enzyme to inhibitor, and the time of preincubation [23]. Inhibition of citrate synthase by propionyl-CoA or methylmalonyl-CoA may be important under certain physiological conditions, and could explain the keto-acidosis and general metabolic disturbances observed in patients with an inborn defect of methylmalonyl-CoA mutase or with vitamin B_{12} deficiency [24, 25].

The suggestion has been made [2] that differences measured in kinetic parameters between rat liver and pig heart citrate synthase reflected differences in metabolic roles of the enzyme in liver and heart. A recent comparison shows, however, that the citrate synthase from rat heart and rat liver are immunologically distinguishable and exhibit comparable inhibition by ATP [18]. The identity of the control features of the enzyme between tissues and species was supported by the fact that the K_m for acetyl-CoA and the K_i for succinyl-CoA for the rat liver enzyme are identical to the respective parameters for the beef heart enzyme within the limits of experimental error (table 1).

The present kinetic studies with purified citrate from beef heart and rat liver provide experimental proof for the postulate of coordination of citric acid cycle flux by alterations of the intramitochondrial succinyl-CoA content [12]. Since succinyl-CoA levels are influenced by the intramitochondrial ATP/ADP ratio via the phosphorylation state of the guanine nucleotides, feedback control to citrate synthase by succinyl-CoA will be strictly energy dependent. Although the K_i for succinyl-CoA (0.14 mM) is well below the reported range of mitochondrial succinyl-CoA concentrations, 0.3 to 1.4 mM [12, 13], the K_m for acetyl-CoA (8 μM) is also much less than the intramitochondrial acetyl-CoA concentrations, 0.1 to 1.0 mM [12–14]. These findings are consistent with the observation that the inhibition of citrate synthase by succinyl-CoA occurs only at low acetyl-CoA concentrations [12]. Inhibition by succinyl-CoA, unlike that of ATP, is unaffected by Mg^{2+}. Because of the high stability constant for the ATP-Mg complex [26], essentially all the ATP exists as this form at the molar ratios of Mg^{2+} to ATP greater than unity which prevail in the mitochondrial matrix [12]. Regulation of citric acid cycle flux by a direct ATP inhibition of citrate synthase, therefore, would appear unlikely under physiological conditions. Inhibition of citrate synthase by succinyl-CoA rather than ATP probably accounts for the increased rate of ketogenesis observed by Krebs [27] upon addition of α-ketoglutarate to rat liver homogenates, and for the respiratory inhibitions observed with isolated rat liver and rat brain mitochondria after addition of ATP in the presence of oligomycin [3, 28].

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