Regulatory Studies on Citrate Synthase in Candida 107, an Oleaginous Yeast

By CHRISTOPHER A. BOULTON* AND COLIN RATLEDGE
Department of Biochemistry, University of Hull, Hull HU6 7RX

(Received 23 May 1980; revised 8 July 1980)

The kinetic properties of citrate synthase from Candida 107 were examined. Adenine nucleotides were inhibitory to enzyme activity, the order of efficacy being ATP > ADP > AMP. Inhibition by ATP was competitive with respect to acetyl-CoA and mixed with respect to oxaloacetate. Combinations of adenine nucleotides giving simulated energy charge values were also inhibitory, though the total adenine nucleotide concentration was of greater significance than the relative proportions of each in determining the degree of inhibition. When Mg²⁺ was added at a concentration sufficient to saturate the adenine nucleotides, the inhibition was almost entirely relieved. The apparent absence of any rigorous control of citrate synthase by adenine nucleotides in oleaginous micro-organisms is consistent with previous observations that the flow of carbon through the glycolytic and pentose phosphate pathways to pyruvate and thence to citrate should be uninterrupted during the process of lipid accumulation.

INTRODUCTION

Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating); EC 4.1.3.7] is regarded as a probable control-point in the citric acid cycle and a variety of metabolites, including ATP and NADH, have been nominated as potential effectors (for review, see Weitzman & Danson, 1976). As both NADH and ATP can be regarded as end-products of the citric acid cycle both would appear to be involved in examples of feedback control mechanisms, particularly as citrate synthase can be viewed as the first reaction of the cycle. Atkinson (1968) considered that the relative proportions of adenine nucleotides are of regulatory significance in many aspects of metabolism and he has suggested that citrate synthase is such an enzyme responsive to changes in the 'energy charge' of a cell, energy charge being defined as (ATP + ½ADP)/(ATP + ADP + AMP).

Our interest in the regulation of citrate synthase has arisen from our studies into the reasons for lipid accumulation in certain yeasts and moulds. Central to any hypothesis advanced to account for continued lipid synthesis (Botham & Ratledge, 1979) is the need to see that there is an uninterrupted flow of carbon from the principal growth substrate to acetyl-CoA. As we have no reason to believe that oleaginous yeasts have any major change in the localization of key enzymes converting pyruvate, produced by glycolysis or the pentose phosphate cycle, to acetyl-CoA (C. A. Boulton, unpublished work), the flow of carbon from pyruvate must be into the mitochondria there to give both acetyl-CoA and, by carboxylation, oxaloacetate from which citrate is produced by citrate synthase. When the appropriate metabolic conditions prevail, citrate is transported out of the mitochondria into the cytosol where it is cleaved into acetyl-CoA and oxaloacetate by the action of ATP: citrate lyase, an enzyme so far only found in oleaginous micro-organisms (C. A. Boulton, unpublished work). As citrate synthase is involved in this sequence and is known
to be an enzyme susceptible to the regulatory influence of adenine nucleotides in other tissues (Shepherd & Garland, 1969), we have analysed its kinetic properties in some detail.

METHODS

Growth of organism. Candida 107 was grown in a 1 l chemostat (LHE 500 series II, LH Engineering, Stoke Poges, Bucks.) using a glucose/salts, nitrogen-limiting medium as previously described (Botham & Ratledge, 1979). The organism was grown at 30°C at a dilution rate of 0.05 h⁻¹ and the culture was maintained at pH 5.5 by the automatic addition of NaOH.

Preparation of extracts. Yeast was taken from the chemostat, harvested and washed as previously described (Botham & Ratledge, 1979), resuspended in 50 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 7.5, to give 0.4 g (wet wt) ml⁻¹, then disrupted by passage through a pre-cooled French pressure cell at 35 MPa. Whole yeast and debris were removed by centrifuging the disrupted material at 45000 g and 4°C for 30 min. Unsedimented lipid was removed by filtering the supernatant through Whatman no. 1 paper.

Enzyme assay. Citrate synthase was assayed by the method of Srere et al. (1963). The reaction mixture contained, in 1 ml: Tris/HCl buffer, pH 8.0, 100 mM; 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; made up in 20 mM-Tris/HCl, pH 8.0, 0.25 mM; oxaloacetate (prepared each day and neutralized prior to use), 200 µM; acetyl-CoA (Sigma), 100 µM. Assays were performed at 25°C using a blank with all the components but extract present. Reactions were started by adding oxaloacetate, and the appearance of the mercaptide ion, formed from DTNB and CoA, was followed by measuring A₄₁₅ in a recording spectrophotometer.

Kinetic analyses. Assays were performed with substrate concentrations as indicated in the relevant Results section.

Enzyme purification. In a typical purification, 500 ml culture was removed from the chemostat and, after harvesting and washing, this yielded 15 to 20 g (wet wt) cells. The extract was prepared as described above and all procedures were carried out at 4°C. Solid (NH₄)₂SO₄ was added to the stirred extract over 15 min to give 55% saturation. After a further 15 min the mixture was centrifuged at 30000 g for 10 min and the precipitate was discarded. The supernatant was taken to 80% saturation in a similar manner and the resulting precipitate was dissolved in a minimum volume of 10 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 7.5. The (NH₄)₂SO₄ fraction was applied to a column (2.5 × 40 cm) of Sephadex G-150 which had been equilibrated in 10 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 7.5. After elution with similar buffer, the most active fractions were combined and applied to a column (3 × 8 cm) of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) equilibrated in 10 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 7.5. The enzyme was eluted with 200 ml of a linear gradient of KH₂PO₄/Na₂HPO₄ buffer, pH 7.5, from 10 to 200 mM. Active fractions were combined and stored at -15°C until needed.

Protein was determined by the method of Lowry using dried crystalline bovine albumin as standard.

Determination of enzyme purity. A sample of the purified material was concentrated 10-fold with an Amicon micro-volume ultrafiltration cell, fitted with a PM10 membrane (Amicon Corporation, Lexington, Mass., U.S.A.). Samples of the concentrate, containing approx. 100 µg protein, were developed at 4°C on 7.5% (w/v) polyacrylamide gels, according to the method of Davis (1964), in 0.04 M-Tris/glycine buffer, pH 8.3, at 2 mA per tube. Protein was detected by staining for 1 h in 1% (w/v) Naphthalene Black dissolved in 7% (v/v) acetic acid. The gels were destained by irrigation in 20% (v/v) acetic acid. Activity was detected by cutting an unfixed gel, which had been rapidly frozen in powdered solid CO₂, into 1 mm slices and immersing the slices in the standard assay mixture; the enzyme was located by the appearance of the yellow mercaptide ion. As a control, a gel was treated in a similar manner but without oxaloacetate in the assay mixture.

RESULTS

After a typical purification of citrate synthase (Table 1), polyacrylamide disc gel electrophoresis indicated that the enzyme was 10 to 15% pure. Although this was a modest purification judging from other achievements (e.g. Srere, 1969; Parvin, 1969), the preparation was free from contaminating activities, such as acetyl-CoA deacylase, malate dehydrogenase, aconitase and ATP:citrate lyase, which might interfere with the assay. The preparation was therefore judged suitable for further characterization of the enzyme and was used for all subsequent work.

The reaction velocity of the enzyme varied only slightly from pH 7.4 to 9.0, in 0.1 M-Tris/HCl buffer; pH 8.0 was chosen for subsequent assays.
Table 1. A typical purification of citrate synthase from Candida 107

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Activity (µmol min⁻¹ ml⁻¹)</th>
<th>Protein (mg ml⁻¹)</th>
<th>Specific activity (µmol min⁻¹ mg⁻¹)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>45-0</td>
<td>5-3</td>
<td>8-1</td>
<td>0-66</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate fraction</td>
<td>2-9</td>
<td>63-6</td>
<td>15-2</td>
<td>4-2</td>
<td>6</td>
<td>77</td>
</tr>
<tr>
<td>Sephadex eluate</td>
<td>43-0</td>
<td>3-5</td>
<td>0-5</td>
<td>7-0</td>
<td>11</td>
<td>63</td>
</tr>
<tr>
<td>Hydroxylapatite eluate</td>
<td>46-0</td>
<td>2-9</td>
<td>0-13</td>
<td>22-3</td>
<td>34</td>
<td>56</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of adenine nucleotides [ATP (●), ADP (○), AMP (▲)] on the activity of citrate synthase. The enzyme was assayed with (a) oxaloacetate at 50 μM and acetyl-CoA at 5 μM and (b) oxaloacetate at 5 μM and acetyl-CoA at 50 μM. Activities are shown relative to the activity in the absence of adenine nucleotides [0-16 and 0-12 µmol min⁻¹ (mg protein)⁻¹ in (a) and (b), respectively].

Fig. 2. Activity of citrate synthase at various simulated energy charge values [(ATP+½ADP)/(ATP+ADP+AMP)] using total adenine nucleotide concentrations of 0-5 mM (●), 2-5 mM (○) and 5-0 mM (▲). The enzyme was assayed with (a) oxaloacetate at 50 μM and acetyl-CoA at 5 μM and (b) oxaloacetate at 5 μM and acetyl-CoA at 50 μM. Activities are shown relative to the activity with 0-5 mM-AMP [0-14 and 0-11 µmol min⁻¹ (mg protein)⁻¹ in (a) and (b), respectively].

The usual Michaelis–Menten relationship was found to be valid when the initial reaction velocity was measured as a function of both oxaloacetate and acetyl-CoA concentrations. True Km values were calculated to be 0-4 μM for oxaloacetate and 2-0 μM for acetyl-CoA.

**Effect of adenine nucleotides and energy charge**

Adenine nucleotides inhibited the enzyme when assayed with non-saturating concentrations of either substrate; the order of efficacy was ATP > ADP > AMP (Fig. 1). The
degree of inhibition was greater at non-saturating acetyl-CoA concentrations than with non-saturating concentrations of oxaloacetate.

The kinetics of ATP inhibition of the enzyme were competitive with respect to acetyl-CoA, the $K_i$ being 0.14 mM, and mixed (competitive and non-competitive) with respect to oxaloacetate.

Initial velocity measurements were also made with various combinations of adenine nucleotides so as to effect different energy charge values. As adenylate kinase was not present in these preparations an intermediate value of 1.2 for its equilibrium constant was assumed and proportions of each nucleotide, at each energy charge value, were then calculated according to Bomsel & Pradet (1968). Three different total concentrations of the adenine nucleotides were used. Although inhibition of activity was always highest at a high energy charge value (Fig. 2), there was a considerable difference in the degree of inhibition produced by the three concentrations of nucleotides. The pattern of inhibition (Fig. 2) was similar whether acetyl-CoA or oxaloacetate was the non-saturating substrate.

**Effect of Mg$^{2+}$ on the pattern of inhibition by adenine nucleotides**

The inhibition of citrate synthase produced by ATP (cf. Fig. 1) was considerably alleviated when Mg$^{2+}$ was included in the assay (Fig. 3). In the absence of ATP, Mg$^{2+}$ was inhibitory to the reaction, the degree of inhibition being proportional to the concentration of Mg$^{2+}$ added. However, with the highest concentration of Mg$^{2+}$ used (2.5 mM) only minimal inhibition was produced even by the highest concentration of ATP (2.0 mM).

The alleviatory effect of Mg$^{2+}$ was further seen with various combinations of adenine nucleotides simulating different energy charge values (Fig. 4). The data in Fig. 4 clearly indicated that the energy charge did not exert a controlling influence on activity when Mg$^{2+}$ was present and, as noted previously (Fig. 2), an increase in total adenine nucleotide concentration resulted in a decrease in activity.

**Effect of nicotinamide nucleotides and other metabolites**

Citrate synthase, assayed with either acetyl-CoA or oxaloacetate as the limiting substrate, was inhibited only slightly (about 10%) by NAD(P)$^+$ at 1 mM. NADH at the same concentration was more inhibitory, giving about 20% inhibition with each assay system. NADPH at 1 mM produced 34% inhibition when oxaloacetate was the limiting substrate but gave 50% inhibition when acetyl-CoA was limiting. Citrate, DL-isocitrate, pyruvate, 2-oxoglutarate, L-malate and phosphoenolpyruvate, each at 1 mM, produced no significant alteration in enzyme activity.
Fig. 4. Activity of citrate synthase at various simulated energy charge values in the presence of Mg$^{2+}$. Total adenine nucleotide concentrations were 0.5 mM (a), 2.5 mM (b) and 5.0 mM (c). Mg$^{2+}$ concentrations were 0 mM (●), 2.5 mM (○) and 5.0 mM (▲). The enzyme was assayed with oxaloacetate at 50 μM and acetyl-CoA at 5 μM. Activities are shown relative to the activity in the absence of Mg$^{2+}$ but in the presence of 0.5 mM-AMP [0.14 μmol min$^{-1}$ (mg protein)$^{-1}$].

DISCUSSION

Our results have shown that the kinetic properties of citrate synthase from Candida 107 are similar to those of Saccharomyces cerevisiae (Hathaway & Atkinson, 1965). The $K_m$ for acetyl-CoA was 2.0 μM in both yeasts, which was considerably lower than for enzymes from other sources (see Shepherd & Garland, 1969). The inhibition by ATP had a $K_i$ of 0.14 mM with Candida 107 and 0.11 mM with S. cerevisiae and in each case was competitive with acetyl-CoA, but in S. cerevisiae the inhibition was non-competitive with oxaloacetate whereas in Candida 107 the kinetics of inhibition were mixed, although this is probably not a significant difference between the two enzymes.

The inhibition of citrate synthase by ATP was considerable (Figs 1 and 3) and, moreover, combinations of the adenine nucleotides to give simulated energy charge values were also inhibitory towards the activity of the enzyme (Fig. 2). However, we know that in this oleaginous yeast the rate of lipid synthesis is not decreased when cells are starved of nitrogen in order to promote lipid accumulation (Gill et al., 1977), and, therefore, there can be no interruption in the supply of acetyl-CoA. Thus, our finding that citrate synthase would be inhibited under these conditions is paradoxical. To reconcile these observations, we can see two points which previous workers in evaluating the effects of energy charge have failed to take into sufficient account when trying to assess the degree of control over an enzyme.

1. Any discussion of the regulatory significance of energy charge must consider not only the prevailing energy charge but also the total adenine nucleotide concentration in relation to the concentrations of enzyme and substrates. Little information is available as to the precise microenvironment of the enzyme. However, the following points are pertinent. The total adenine nucleotide concentration within the mitochondrion is constant, as passage of ATP into cytosol occurs only via a 1:1 exchange process with AMP (Klingenberg,
1970). Botham & Ratledge (1979) have reported that the total adenine nucleotide pool of this organism is 2 to 4 mM and that the average energy charge varies between 0.1 and 0.6 according to cultural conditions. Although the prevailing energy charge within the mitochondrion may differ from that in the cytosol (Heldt et al., 1972), the concentration of nucleotides may not be significantly different in the two compartments. Hence, simulated energy charges tested against citrate synthase, or any other enzyme, should be in the correct concentration range. However, even an increase from 2.5 to 5 mM in total adenine nucleotide concentration results in a doubling of the inhibitory effect at all energy charge values (Fig. 2). Hence, it is impossible to calculate the *in vivo* activity of the enzyme if neither the energy charge value nor the total adenine nucleotide concentration are known accurately.

2. The effect of simulated energy charge values must take into account that ATP exists predominantly *in vivo* as the magnesium–ATP complex. Knowles (1977) has discussed this point at some length and has pointed out that the concentration of available Mg$^{2+}$ is of great importance in calculating the effects of energy charge. It is probable that intracellular ATP exists not as ATP$^{4-}$ but as a magnesium complex (Burt et al., 1976). Moreover, adenylate kinase, which catalyses the transfer of phosphate between ATP and AMP, is a Mg$^{2+}$-requiring enzyme. It seems reasonable to suppose, therefore, that Mg$^{2+}$ will be part of any interaction between adenine nucleotides and citrate synthase. When Mg$^{2+}$ was included in enzyme assays there was a marked alleviation of the inhibitory effects of ATP and at high simulated energy charge values (Figs 3 and 4). Indeed, it was difficult to demonstrate much inhibition due solely to the nucleotides if comparison was made to the activity of the enzyme determined in the presence of excess Mg$^{2+}$.

We conclude that citrate synthase is unlikely to be strongly inhibited in *Candida* 107 even when the energy charge is approaching its maximum recorded value of 0.55 (Botham & Ratledge, 1979). The relieving effect of Mg$^{2+}$ on the activity is likely to be considerable. This would accord with the view of Weitzman & Hewson (1973) who found that the inhibition *in vitro* of citrate synthase in *S. cerevisiae* by adenine nucleotides was not observed when the enzyme was assayed *in situ* using cells made permeable with toluene. Although interpretation of such results is often equivocal, our findings would support the view that citrate synthase is not an enzyme susceptible to rigours or severe regulatory controls.

This work was supported by a research grant from the A.R.C., AG 21/32.

**References**


