Activities of Citrate Synthase and other Enzymes of *Acetobacter xylinum* *in situ* and *in vitro*

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The activities of a number of enzymes, extracted from *Acetobacter xylinum*, that are involved in carbohydrate metabolism may be accounted for *in situ* in permeabilized cells. The kinetic properties of citrate synthase and glycerokinase observed *in vitro* are also retained *in situ*. So is the regulatory sensitivity of these enzymes. Both *in vitro* and *in situ*, (a) citrate synthase, in contrast with the enzyme from other Gram-negative bacteria, is inhibited by ATP and is insensitive to NADH, and (b) glycerokinase is inhibited by fructose diphosphate and the ratio of its activities towards glycerol and dihydroxyacetone is the same.

The approach that utilizes measurements of activities *in situ* in permeabilized cells for the study of intracellular enzymes in the macromolecular environment prevailing *in vivo* has been proposed as an important way to ascertain whether the behaviour of enzymes studied *in vitro* also applies *in vivo* (Reeves & Sols, 1973; Serrano *et al.*, 1973; Weitzman, 1973). The citrate synthase of yeast is one of the enzymes reported to differ *in situ* from the isolated enzyme, most importantly in this case, in being insensitive to inhibition by ATP (Weitzman & Hewson, 1973). The citrate synthase isolated and purified from *Acetobacter xylinum* extracts was shown (Swissa & Benziman, 1973, 1976) to be inhibited by ATP and to be insensitive to NADH, thus differing from the enzyme isolated from other Gram-negative bacteria. It has been suggested that the flux through the citric acid cycle in *A. xylinum* is regulated by modulation of citrate synthase activity in response to the energy state of the cells (Swissa & Benziman, 1973, 1976).

In this present communication we report our studies on the behaviour of citrate synthase and another regulatory enzyme, glycerokinase (Weinhouse & Benziman, 1975), in permeabilized *A. xylinum* cells. The results indicate that the kinetic properties and regulatory sensitivity of both enzymes observed *in vitro* are retained *in situ*.

Materials and Methods

Cells of *A. xylinum* were grown on succinate and harvested after 24 h as previously described (Benziman, 1969a).

Cell-free extracts were prepared by sonication. Cells suspended to a final concentration of 15 mg (dry wt.)/ml in 50 mM-Tris/H₂SO₄ buffer, pH 7.4, were treated for 15 min in a Raytheon model DFI magneto-restrictive oscillator at 200 W and 10 kHz. The extract was centrifuged in the cold at 10000g for

15 min and the supernatant fluid was used for determination of enzyme activities.

Cells were permeabilized by treatment with toluene. Cells suspended as indicated above were chilled and vigorously agitated with a Vortex mixer. While agitation was continued 0.01 vol. of toluene was added and agitation continued for a further 1 min. The cells were then collected by centrifugation and resuspended in 50 mM-Tris/H₂SO₄ buffer, pH 7.4. The suspension of toluene-treated cells, when kept in ice, retained the activity of all the enzymes tested for several hours.

Citrate synthase activity was assayed with 5,5'-dithiobis-(2-nitrobenzoic acid) (Swissa & Benziman, 1973, 1976; Srere *et al.*, 1963). Glycerokinase activity was determined by the formation from [¹⁴C]glycerol of glycerol 3-phosphate, which was adsorbed on discs of DEAE-cellulose filter paper and then its radioactivity counted, and dihydroxyacetone kinase activity by measuring the oxidation of NADH in a reaction coupled with glycerol 3-phosphate dehydrogenase (Weinhouse & Benziman, 1975; Torner, 1975). FAD-linked malate dehydrogenase activity was assayed by ferricyanide reduction (Benziman, 1969a), and alcohol dehydrogenase activity by acetaldehyde reduction with NADH (Benziman & Abeliowitz, 1964). The activities of the following enzymes were assayed as described in the respective references: NAD- and NADP-linked isocitrate dehydrogenases (Cox, 1969); glucokinase and fructokinase (Benziman & Rivetz, 1972); pyruvate kinase (Benziman, 1969b); pyruvate, phosphate dikinase (Benziman & Palgi, 1970); phosphoenolpyruvate carboxylase (Benziman, 1969c).

Results

Treatment of cell suspensions of *A. xylinum* with 0.01 vol. of toluene for 1 min permeabilized the cells and made a variety of enzymes involved in carbo-

Table 1. Activities of enzymes in cell-free extracts and *in situ* in permeabilized cells of *A. xylinum*

Enzymes were assayed as described in the Methods and Materials section. Enzyme units are expressed as the amount of enzyme transforming 1 μ mol of substrate/min under the assay conditions described.

Enzyme	Activity (munits/mg dry wt. of cells)	
	<i>In situ</i>	Extract
Citrate synthase	330	380
Glycerokinase	34	30
	(glycerol)	(glycerol)
	21	19
	(dihydroxy-acetone)	(dihydroxy-acetone)
NAD-linked isocitrate dehydrogenase	360	260
NADP-linked isocitrate dehydrogenase	260	300
Glucokinase	140	120
Fructokinase	40	30
Pyruvate,phosphate dikinase	17	20
Pyruvate kinase	8	6
Phosphoenolpyruvate carboxylase	8	6
Alcohol dehydrogenase	38	45

hydrate metabolism accessible to external substrates. Supernatant fluids obtained after centrifugation of such treated cells were devoid of any detectable activity of the enzymes tested, thus indicating that the enzymes were retained within the boundaries of the cells. Table 1 compares the activities of the various enzymes tested in toluene-treated preparations with those found in cell-free extracts: the values obtained in both cases are in good agreement.

Treatment of cells with toluene/ethanol (1:4, v/v) mixture (cf. Serrano *et al.*, 1973; Weitzman & Hewson, 1973), either in the cold or at room temperature, resulted in the leaking of part of the activities into the external medium.

To test the effect of the concentrations of acetyl-CoA and oxaloacetate on citrate synthase activity, the assay method was modified so that the non-varied component of the reaction was present at a saturating concentration and the concentration of the substrate under examination was varied. Lineweaver-Burk plots for citrate synthase activity of toluene-treated cells were linear and identical with those obtained for the purified enzyme. The K_m values for oxaloacetate for the enzyme in the two conditions were identical (9 μ M) and the values for acetyl-CoA were similar (18 μ M and 25 μ M for the isolated enzyme and toluene-treated cells respectively). It was also found that the K_m of either substrate was independent of the concentration of the other.

Fig. 1. Inhibition by ATP of citrate synthase activity in toluene-treated *A. xylinum* cells

Reaction mixtures (1.0ml) contained: 100 μ mol of glycine/NaOH buffer (pH 8.4), 0.25 μ mol of freshly prepared oxaloacetate, 0.1 μ mol of 5,5'-dithiobis-(2-nitrobenzoic acid), 2 μ l of toluene-treated cell suspension, the indicated concentrations of acetyl-CoA and (1) no ATP, (2) 0.5 μ mol of ATP, (3) 2.0 μ mol of ATP or (4) 4.0 μ mol of ATP. Velocities were measured at 420nm, and are expressed as $\Delta E_{412}/\text{min}$. K_i values for ATP were 1.5mM with all the concentrations of ATP tested.

ATP, which has been shown to inhibit the purified *A. xylinum* citrate synthase, similarly inhibited this enzyme *in situ*. Inhibition curves at constant ATP concentration and various acetyl-CoA concentrations are given in Fig. 1. The results show that ATP inhibition is competitive with respect to acetyl-CoA with a K_i of 1.5mM. These results are very similar to those obtained with the purified enzyme (Swissa & Benziman, 1973, 1976). Unlike the citrate synthase from other Gram-negative bacteria (Weitzman & Jones, 1968), the enzyme isolated from *A. xylinum* is not sensitive to inhibition by NADH. When the effect of NADH was similarly examined with permeabilized cells, no inhibition was observed even at an NADH concentration of 5mM.

Another enzyme examined *in situ* was glycerokinase. This enzyme, which was extensively purified from *A. xylinum* extracts, was shown to catalyse the phosphorylation by ATP of both glycerol and dihydroxyacetone and to be strongly inhibited by fructose diphosphate (Weinhouse & Benziman, 1975). The glycerokinase/dihydroxyacetone kinase activity ratio measured *in situ*, 1.6, was the same as that previously obtained throughout the purification of the enzyme. As with the purified enzyme, reaction rates with the enzyme *in situ* were hyperbolic functions of glycerol, dihydroxyacetone and ATP concentra-

tions, the corresponding K_m values for the various substrates being identical for the enzyme in the two conditions (glycerol, 0.5 mM; dihydroxyacetone, 5 mM; ATP, 0.5 mM). Fructose diphosphate inhibition of both kinase activities was retained *in situ*. The inhibition, which followed classical kinetic patterns, was competitive with respect to ATP with a K_i value of 0.02 mM, identical with that obtained with the purified enzyme.

Discussion

The citrate synthase of *A. xylinum* studied *in situ* exhibits properties very similar to those found with the enzyme isolated from extracts of the cells. This finding is in contrast with the results obtained by Weitzman & Hewson (1973), who reported the citrate synthase of yeast *in situ* to differ considerably from the isolated enzyme in exhibiting a much higher K_m for acetyl-CoA, and most importantly in being insensitive to ATP inhibition. Since it is assumed that the true physiological behaviour of enzymes *in vivo* is more closely approximated by their behaviour *in situ* than by that exhibited in cell-free extracts, Weitzman & Hewson (1973) interpreted their results as suggesting that ATP inhibition of citrate synthase, in yeast and possibly in some other types of cells, is not a physiologically significant regulatory mechanism. With *A. xylinum*, however, the results obtained suggest rather that citrate synthase at concentrations found *in vivo* retains its sensitivity to ATP inhibition. They support the view suggested previously (Swissa & Benziman, 1973, 1976) that such inhibition, at least in *A. xylinum*, may be of physiological significance as the primary means by which the entry of acetate into the citric acid cycle is kept in step with the energy state of the cells.

The properties of the glycerokinase/dihydroxyacetone kinase *in situ*, especially with regard to the ratio of activities with the two substrates and its inhibition by fructose diphosphate, lend additional support to our former conclusion (Weinhouse & Benziman, 1975) that a single enzyme is responsible for the phosphorylation of both glycerol and dihydroxyacetone and that fructose diphosphate inhibition of this enzyme is physiologically important in the control of glycerol metabolism in *A. xylinum*.

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