Regulation of Autotrophic Metabolism in *Pseudomonas oxalaticus* OX1 Wild-type and an Isocitrate-lyase-deficient Mutant

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In *Pseudomonas oxalaticus* the activity and synthesis of the Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) are regulated by inactivation and endproduct repression, respectively. Phosphoenolpyruvate (PEP) has been suggested to function as a signal molecule for the latter control system. During growth of the organism in carbon-source-limited continuous cultures with various ratios of acetate and formate in the feed, the RuBisCO levels varied considerably, but no correlation was observed with the intracellular concentrations of PEP. To study whether the repression exerted by acetate utilization was dependent on the synthesis of glycolytic intermediates from this compound, an acetate-negative mutant defective in isocitrate lyase was isolated and characterized. Clear evidence was obtained that in this mutant acetate is as effective in repressing RuBisCO synthesis as in the wild-type. It therefore appears more likely that acetyl-CoA or a closely related metabolite functions as a signal molecule in the regulation of RuBisCO synthesis.

INTRODUCTION

In the facultative autotroph *Pseudomonas oxalaticus* OX1 the synthesis of RuBisCO, the key enzyme in the Calvin cycle of CO₂ fixation, is highly regulated. During growth of the organism on formate, a substrate which is oxidized to CO₂ by formate dehydrogenases, large amounts of RuBisCO are synthesized. During growth in batch cultures on substrates which allow a rapid, heterotrophic growth mode (e.g. acetate), no enzyme activity is detectable. Addition of formate to the latter cultures results in its utilization as an ancillary energy source, but not in the synthesis of RuBisCO (Dijkhuizen *et al.*, 1978).

When *P. oxalaticus* is grown on a mixture of formate and acetate in carbon-limited continuous cultures, repression of RuBisCO synthesis is less severe, and dependent on the ratio of the two substrates in the feed and the dilution rate (Dijkhuizen & Harder, 1979). To explain these phenomena, it was suggested that the synthesis of RuBisCO in *P. oxalaticus* is regulated via an endproduct repression mechanism in which an intermediate of the central metabolism of the cell may function as a signal molecule (Dijkhuizen & Harder, 1984). Similar conclusions were reached in studies on other facultatively autotrophic bacteria (Bowien *et al.*, 1987; Tabita, 1988).

Further evidence for the identity of the signal molecule involved was obtained in experiments with the hydrogen bacterium *Alcaligenes eutrophus* (Reutz *et al.*, 1982; Im & Friedrich, 1983). This organism metabolizes fructose via the Entner–Doudoroff pathway. Following the introduction of metabolic blocks in the glycolytic part of the Entner–Doudoroff pathway, cells of the organism incubated in fructose mineral medium possessed RuBisCO at levels equivalent

Abbreviations: ICL, isocitrate lyase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PEP, phosphoenolpyruvate.

to those normally encountered only in autotrophically grown cells. A reduced ability to convert fructose into 2-phosphoglycerate and PEP, metabolites which may be considered as endproducts of the Calvin cycle, thus appeared to result in a release of feedback repression in *A. eutrophus*. The degree of repression was proposed to be correlated with the intracellular concentration of PEP.

It was the objective of the present study to obtain more detailed information about the regulation of autotrophic CO₂ fixation in *P. oxalaticus* by acetate. Assimilation of acetate via the glyoxylate cycle results in the production of glycolytic intermediates such as PEP. The observed regulation of RuBisCO synthesis in cells growing in the presence of acetate thus might depend on the intracellular levels of this signal molecule.

METHODS

Organism and growth conditions. *Pseudomonas oxalaticus* OX1 wild-type, its maintenance and the mineral salts media used in batch and continuous cultures have been described previously (Dijkhuizen & Harder, 1975; Dijkhuizen *et al.*, 1977), as have the experimental conditions of growth on single and mixed substrates in batch (Dijkhuizen *et al.*, 1978) and continuous cultures (Dijkhuizen *et al.*, 1977; Dijkhuizen & Harder, 1979). During growth on formate in a batch fermenter with a working volume of 3 litres, the pH was kept constant by automatic titration with formic acid (25%, w/v) (Knight *et al.*, 1978).

Preparation of cell-free extracts and enzyme assays. Cell-free extracts were prepared according to Dijkhuizen *et al.* (1978). Enzyme measurements were made in a Hitachi model 100-60 spectrophotometer at 30 °C. In all assays the observed rate was linear for at least 3 min and was proportional to the amount of cell-free extract added. The following enzymes were assayed according to published procedures: isocitrate lyase, EC 4.1.3.1 (Dixon & Kornberg, 1959); ribulose-1,5-bisphosphate carboxylase, EC 4.1.1.39, and NAD-dependent formate dehydrogenase, EC 1.2.1.2 (Dijkhuizen *et al.*, 1978); isocitrate dehydrogenase (NADP-dependent), EC 1.1.1.42 (Levering & Dijkhuizen, 1985). Citrate synthase, EC 4.1.3.7, was assayed at 412 nm by following the rate of reduction of 5,5'-dithiobis-(2-nitro-benzoate) (DTNB) in the presence of acetyl-CoA and oxaloacetate (Srere, 1969). The reaction mixture (1 ml) contained: Tris/HCl buffer, pH 8.0, 50 µmol; DTNB, 0.1 µmol; acetyl-CoA, 0.3 µmol; and extract. The endogenous rate of DTNB reduction, due to the presence of acetyl-CoA deacylase activity was followed for 3 min. The reaction was started by the addition of 1 µmol oxaloacetate. The molar extinction coefficient of DTNB at 412 nm was taken as $13.6 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$.

Determination of intracellular PEP concentrations. The methods used for rapid sampling of cells from continuous cultures, extraction of metabolites and the pyruvate-kinase-dependent conversion of PEP and ADP into pyruvate and ATP are those described by Otto (1984). The amount of ATP formed was determined according to Otto *et al.* (1984), using the luciferine-luciferase system and measuring the ATP-dependent light emission in an Aminco Chem Glow Photometer. The reaction mixture (200 µl) contained (final concentrations): 50 mM-Tris/acetate, pH 7.75; 1.5 mM-EDTA; 0.075% (w/v) bovine serum albumin; 10 mM-magnesium acetate; 35 µM-luciferine and 5000 units luciferase (Boehringer Mannheim). Duplicate samples were taken from at least two individual steady states at each ratio of acetate and formate in the feed. The PEP levels in each sample were measured in triplicate. At each ratio of acetate and formate in the feed less than 10% variation was observed in the PEP levels in the separate samples.

Isolation of acetate-negative mutants. Transposon mutagenesis was carried out using a *P. oxalaticus* wild-type strain carrying plasmid pNJ5000::Tn7. This vector can be stably maintained only in recombination-deficient strains and during growth in the presence of the antibiotics tetracycline (15 µg ml⁻¹) and trimethoprim (20 µg ml⁻¹), selecting for the resistance markers coded for by the plasmid and transposon, respectively (Grinter, 1983). Mutants were induced by growing this strain in the absence of antibiotics in fructose mineral medium for 48 h. The cells were subsequently harvested by centrifugation, washed once and diluted 100-fold in mineral medium without carbon source. After 24 h starvation, acetate (10 mM) and ampicillin (50 µg ml⁻¹) were added to selectively kill cells able to grow on acetate. After 12 h incubation the cells were harvested, thoroughly washed, and grown in fructose mineral medium followed by a second, identical enrichment cycle. Samples were plated out on fructose mineral agar containing trimethoprim and acetate-negative mutants selected by replica plating of the colonies obtained on acetate agar.

Other analytical methods. The maximum capacity of washed cell suspensions to oxidize formate and acetate ($Q_{O_2}^{\max}$) was determined according to Dijkhuizen & Harder (1975). Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard. Culture dry weight values were determined with a Total Carbon Analyzer (Beckman, model 915A), connected to an Infrared Analyzer (Beckman, model 865).

RESULTS

Transition from autotrophic to heterotrophic growth conditions

The effects of acetate on the autotrophic potential of *P. oxalaticus* were studied in more detail by transferring a formate-limited continuous culture of the organism to acetate-limiting conditions (Fig. 1). The transition from autotrophic to heterotrophic growth proceeded smoothly, without accumulation of acetate, and after one to two volume changes the culture slowly reached a new steady state (Fig. 1*a*). In the early hours of the experiment the isocitrate lyase (ICL) activity rapidly increased from a low basal level. In the same period the citrate synthase activity more than doubled, reflecting the more important role of the tricarboxylic acid cycle in energy generation during growth on acetate than on formate. The isocitrate dehydrogenase activity on the other hand strongly fluctuated during the adaptation period, but remained on average at the same level (Fig. 1*b*). Interestingly, the activities of the NAD-dependent formate dehydrogenase and RuBisCO (Fig. 1*a*) decreased rapidly following the substrate transition. After correcting the data for the changes that occurred in cell density, it became clear that the drop in the activities of these enzymes was in fact considerably faster than the wash-out rate. It therefore has to be concluded that under these conditions the enzymes became inactivated.

Intracellular levels of PEP during steady state growth conditions

A possible direct involvement of PEP in the control of RuBisCO synthesis was studied by measuring the intracellular levels of this metabolite in *P. oxalaticus*, grown under steady state conditions in chemostat cultures. As reported previously (Dijkhuizen & Harder, 1979), the synthesis of RuBisCO in this organism became increasingly repressed when the acetate/formate ratios in the feed were increased (Table 1). Addition of acetate stimulated the synthesis of ICL, allowing assimilation of this C₂ compound via the glyoxylate cycle, thus resulting in increased biomass levels. No significant changes in the intracellular concentrations of PEP, however, were observed under these carbon-limiting steady state conditions. This was not even the case when the organism was grown on acetate alone, which resulted in a complete switch-off of RuBisCO synthesis (Table 1). On the basis of these observations it became unlikely that PEP functioned directly as a signal molecule in the regulation of RuBisCO synthesis. This raised the question whether the repression exerted by acetate metabolism on RuBisCO synthesis was dependent on its assimilation via the glyoxylate cycle, resulting in generation of oxaloacetate and glycolytic intermediates.

Isolation of isocitrate-lyase-negative mutants

After transposon mutagenesis 2000 trimethoprim resistant colonies were tested, seven of which were unable to grow on acetate agar, but had retained their ability to grow on fructose, lactate, succinate or formate. We subsequently observed that lactate-grown cells of wild-type *P. oxalaticus* possessed ICL activity [45 nmol min⁻¹ (mg protein)⁻¹], although the enzyme is not required for growth on this substrate. Following growth on lactate three of the acetate-negative strains were found to be affected in ICL synthesis. One of these mutants, strain ACE 3, turned out to be very stable and completely lacked ICL activity. This strain, which was still able to oxidize acetate when this compound was added to cultures growing exponentially on fructose (data not shown), was selected for further studies.

Growth of strain ACE 3 on acetate plus formate

Initially, we considered it theoretically possible that an ICL-negative strain would be able to grow autotrophically on acetate, by combining energy generation from acetate and CO₂ assimilation via the Calvin cycle. Acetate-negative mutants such as strain ACE 3, however, could be isolated in a straight-forward manner. Incubation of fructose-pregrown cells of strain ACE 3 in acetate (10 mM) medium nevertheless resulted in a linear increase in the optical density of the culture, with a doubling time of 20 h (data not shown). No evidence could be obtained that this was based on autotrophic CO₂ fixation as the activity of RuBisCO (and ICL)

Fig. 1. Transition of formate-limited ($S_R = 100$ mM; $D = 0.10$ h⁻¹) cells of *P. oxalaticus* OX1 to acetate-limiting ($S_R = 30$ mM; $D = 0.10$ h⁻¹) growth conditions. (a) Optical density (●); NAD-dependent formate dehydrogenase (Δ); RuBisCO (▲). (b) ICL (▽); isocitrate dehydrogenase (▼); citrate synthase (○). Enzyme activities are given as nmol min⁻¹ (mg protein)⁻¹.

Table 1. Enzyme activities, dry wt values and intracellular PEP values in *P. oxalaticus* OX1

P. oxalaticus was grown on formate and acetate in carbon-source-limited culture at $D = 0.10$ h⁻¹.

Substrate concentration in feed (mM)		Dry wt (mg l ⁻¹)	RuBisCo [nmol min ⁻¹ (mg protein) ⁻¹]	ICL [nmol (mg protein) ⁻¹]	PEP [nmol (mg protein) ⁻¹]
Formate	Acetate				
100	0	365	220	0	2.5
100	5	515	120	60	2.9
100	10	640	95	120	2.9
0	30	645	0	150	3.1

remained undetectable [< 5 nmol min (mg protein)⁻¹]. This phenomenon instead appeared to be due to the synthesis from acetate of considerable amounts of storage material, which was identified as polyhydroxybutyrate (data not shown).

The initial characterization of strain ACE 3 showed that it was not affected in formate metabolism, displaying the normal growth rate on formate alone (doubling time 3.5 h). On a mixture of acetate (10 mM) plus formate (15 mM) it nevertheless behaved as described above for acetate alone. Again a linear increase in optical density (doubling time 20 h) was observed, and not only ICL but also RuBisCO and formate dehydrogenase activities remained undetectable (data not shown). These observations suggested that in strain ACE 3 acetate was still able to repress the synthesis of the autotrophic enzymes. Clear confirmation for this was subsequently obtained in experiments with formate-pregrown cells of strain ACE 3 and, for comparison, wild-type *P. oxalaticus* (Figs 2 and 3). Addition of a pulse of acetate (5 mM) to a mid-exponential phase culture of the wild-type strain growing on formate (15 mM) resulted in a decrease in the doubling time from 3.4 to 2.5 h (Fig. 2). The $Q_{O_2}^{\max}$ -acetate and the ICL activities increased almost immediately, whereas the $Q_{O_2}^{\max}$ -formate and RuBisCO activities dropped gradually. The levels of isocitrate dehydrogenase remained approximately constant. In a similar experiment with strain ACE 3, no change in growth rate was apparent following acetate addition (Fig. 3). In this mutant ICL activity remained undetectable throughout the experiment and only relatively low levels of the $Q_{O_2}^{\max}$ -acetate were observed. Whereas the $Q_{O_2}^{\max}$ -formate and isocitrate

Fig. 2

Fig. 3

Fig. 2. Growth of *P. oxalaticus* OX1 wild-type on formate (15 mM; pH control by automatic titration with formic acid, 25%, w/v), and the effects of a pulsed addition (arrow) of acetate (5 mM). (a) Optical density (●); RuBisCO (▲); ICL (▽). (b) $Q_{O_2}^{\max}$ -acetate (■); $Q_{O_2}^{\max}$ -formate (□); isocitrate dehydrogenase (▼). Enzyme activities are given as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Fig. 3. Growth of the ICL-negative mutant strain ACE 3 on formate (15 mM; pH control by automatic titration with formic acid, 25%, w/v), and the effects of a pulsed addition (arrow) of acetate (5 mM). (a) Optical density (●); RuBisCO (▲); ICL (▽). (b) $Q_{O_2}^{\max}$ -acetate (■); $Q_{O_2}^{\max}$ -formate (□); isocitrate dehydrogenase (▼). Enzyme activities are given as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

dehydrogenase activity hardly changed at all, the RuBisCO activities decreased rapidly. Under these batch culture conditions, with a controlled supply of formic acid as the titrant for pH regulation, inactivation of RuBisCO was not evident with either strain. From the data obtained it could be calculated that in both organisms RuBisCO synthesis became repressed completely following acetate addition, i.e. that the decrease in RuBisCO activities could be explained by a redistribution of existing enzyme over newly synthesized cells. It is concluded that in strain ACE 3, which is unable to convert acetate into glycolytic intermediates, the synthesis of RuBisCO is as sensitive to repression by acetate as in the wild-type organism.

DISCUSSION

Growth of *P. oxalaticus* on mixtures of acetate and formate in batch cultures results in a complete switch-off of the energetically expensive CO₂ fixation (Dijkhuizen *et al.*, 1978). In the present study it became clear that, in addition to regulation via repression of enzyme synthesis, the organism also has a mechanism for rapid inactivation of RuBisCO under conditions where its presence is no longer required (Fig. 1). In substrate transition experiments with *Thiobacillus versutus* (Gottschal *et al.*, 1981), RuBisCO inactivation was shown to occur via proteolytic degradation of the enzyme. Recently, Mann & Turner (1987) provided preliminary evidence that RuBisCO activity in *Rhodocrobium vannielii* is regulated via (de)phosphorylation. The precise mechanism involved in *P. oxalaticus* requires further investigation.

The physiological evidence available suggested that, as is the case for other biosynthetic pathways, synthesis of the enzymes of autotrophic CO₂ fixation is controlled by the intracellular

levels of endproducts of the Calvin cycle, with PEP being the strongest candidate for such a signal molecule function (Reutz *et al.*, 1982; Dijkhuizen & Harder, 1984). No correlation, however, was observed between RuBisCO and PEP levels (Table 1). This made it unlikely that PEP functioned directly as a signal molecule. The subsequent isolation of ICL-negative mutants allowed us to assess whether the repressive effect exerted by acetate in *P. oxalaticus* in fact was dependent on its conversion into oxaloacetate (and PEP) via the glyoxylate cycle. The theoretical possibility that in such a mutant energy generation from acetate oxidation could now serve to drive autotrophic CO₂ fixation was tested but no evidence for this could be obtained. This might have been due to the fact that autotrophic CO₂ fixation requires a higher rate of energy generation than could be sustained by acetate alone in the mutant. We therefore attempted to stimulate energy generation by incubating strain ACE 3 in the presence of both acetate and formate. Again, no RuBisCO synthesis occurred and although strain ACE 3 was still able to grow on formate alone with a doubling time of 3.5 h, no stimulation of growth could be observed following formate addition.

An analysis of the effects of pulsed addition of acetate to cultures of strain ACE 3 and the wild-type in the mid-exponential phase of growth on formate subsequently made it clear that in the mutant strain acetate is as strong a source of repression of RuBisCO synthesis as in the wild-type (Figs 2 and 3). On the basis of these observations it is considered more likely that acetyl-CoA, or a metabolite derived from it, is involved as a signal molecule for the regulation of the synthesis of RuBisCO.

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