Pyruvate carboxylase as an anaplerotic enzyme in *Corynebacterium glutamicum*

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The recent discovery that phosphoenolpyruvate carboxylase (PEPCx) is dispensable for growth and lysine production in *Corynebacterium glutamicum* implies that this organism possesses (an) alternative anaplerotic enzyme(s). In permeabilized cells of *C. glutamicum*, we detected pyruvate carboxylase (PCx) activity. This activity was effectively inhibited by low concentrations of ADP, AMP and acetyl-CoA. PCx activity was highest \[45 \pm 5 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}\] in cells grown on lactate or pyruvate, and was about two- to threefold lower when the cells were grown on glucose or acetate, suggesting that formation of PCx is regulated by the carbon source in the growth medium. In cells grown at low concentrations of biotin (< 5 µg l\(^{-1}\)), PCx activity was drastically reduced, indicating that the enzyme is a biotin protein. Growth experiments with the wild-type and a defined PEPCx-negative mutant of *C. glutamicum* on glucose showed that the mutant has a significantly higher demand for biotin than the wild-type, whereas both strains have the same high biotin requirement for growth on lactate and the same low biotin requirement for growth on acetate. These results indicate that (i) PCx is an essential anaplerotic enzyme for growth on glucose in the absence of PEPCx, (ii) PCx is an essential anaplerotic enzyme for growth on lactate even in the presence of PEPCx, and (iii) PCx has no anaplerotic significance for growth on acetate as the carbon source. In support of these conclusions, screening for clones unable to grow on a minimal medium containing lactate, but able to grow on a medium containing glucose or acetate, led to the isolation of PCx-defective mutants of *C. glutamicum*.

**Keywords**: *Corynebacterium glutamicum*, anaplerotic reactions, pyruvate carboxylase, phosphoenolpyruvate carboxylase

INTRODUCTION

Bacteria require anaplerotic reactions for continuous replenishment of the tricarboxylic acid (TCA) cycle with C\(_2\)-dicarboxylic acids used for anabolic purposes, for example amino acid biosynthesis (Kornberg, 1966). In most organisms, the anaplerotic function during growth on glucose is mediated by either phosphoenolpyruvate (PEP) carboxylase (PEPCx; reaction 1) or pyruvate carboxylase (PCx; reaction 2) (Wood & Utter, 1965; Kornberg, 1966; Schobert & Bowien, 1984), and some *Arthrobacter* strains even use the glyoxylate cycle as an anaplerotic sequence for growth on glucose (Krulwich & Pelliccione, 1979).

\[
\text{PEP} + \text{HCO}_3^- \xrightarrow{\text{PEPCx}} \text{Oxaloacetate} + \text{P}_1
\]

\[
\text{Pyruvate} + \text{HCO}_3^- + \text{ATP} \xrightarrow{\text{PCx}} \text{Oxaloacetate} + \text{ADP} + \text{P}_1
\]

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**Abbreviations**: CTAB, \(N\)-cetyl-\(N,\N,\N\)-trimethylammonium bromide; GOT, glutamate-oxaloacetate transaminase; PCx, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCx, PEP carboxykinase; PEPCx, PEP carboxylase; TCA, tricarboxylic acid; WT, wild-type.

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generally been attributed to PEPCx (Kinoshita, 1985; Liebl, 1991). This enzyme is present with relatively high specific activities in all C. glutamicum strains tested (Ozaki & Shio, 1969; Mori & Shio, 1985a; Eikmanns et al., 1989; Jetten et al., 1994; Gubler et al., 1994). From C. glutamicum subsp. flavum, the enzyme was purified and shown to be activated by acetyl-CoA and fructose-1,6-bisphosphate and inhibited by aspartate and 2-oxoglutarate (Mori & Shio, 1985a, b). These regulatory properties, as well as carbon flux studies (Vallino & Stephanopoulos, 1993), suggested a key role of PEPCx as an anaplerotic enzyme, and thus in carbon flow to amino acids derived from the TCA cycle. However, comparison of defined PEPCx-negative mutants with the parent strains of C. glutamicum showed identical growth characteristics on all media tested, and identical specific activities in all C. glutamicum. The cells were grown aerobically as 60 ml cultures in 500 ml baffled Erlenmeyer flasks at 30 °C. Growth was measured as increase in OD₆₀₀.

For isolation of PCx-defective mutants, C. glutamicum R127 cells grown overnight on 2× TY broth were incubated at 25 °C with NMG (N'-methyl-N'-nitro-N-nitrosoguanidine) (0.25 mg ml⁻¹) for 15 min. The cells were then washed twice in 50 mM Tris/HCl buffer, pH 7, resuspended in the same buffer containing 30% (v/v) glycerol and stored at -20 °C until used. To screen for PCx-negative mutants, aliquots of the cell suspension were spread on LB agar and successively replicated on minimal medium agar containing glucose, lactate, pyruvate or acetate as carbon sources.

**PCx assay with permeabilized cells.** PCx activity in permeabilized cells of C. glutamicum was determined by the discontinuous glutamate-oxaloacetate transaminase- (GOT-) coupled assay described by Fisher & Magasanik (1984). For this assay, cells were grown in minimal medium to the exponential growth phase, washed once in 20 ml 50 mM Tris/HCl buffer, pH 6.3, containing 50 mM NaCl, resuspended to an OD₆₀₀ of 150 in 100 mM HEPES buffer, pH 7.5, containing 20% (v/v) glycerol and then frozen at -20 °C. For permeabilization, the frozen cells were thawed slowly on ice and then mixed with a solution of 10% (w/v) CTAB (N-cetyl-N,N,N-trimethylammonium bromide) to give a final concentration of 0.3 %. This cell suspension was used directly to assay for PCx by the formation of oxaloacetate from pyruvate, HCO₃⁻ and ATP, and subsequent conversion of the oxaloacetate to aspartate with GOT. The standard reaction mixture consisted of 100 mM Tris/HCl, pH 7.3, 25 mM NaHCO₃, 20 mM pyruvate, 4 mM ATP, 2 mM glutamate, 20 μM pyridoxal phosphate and 2 units (U) of pig heart GOT in a final volume of 1 ml. The reaction was started by adding up to 30 μl of the cell suspension. After incubation of the reaction mixture at 30 °C for 60, 90 or 120 s, the reaction was terminated by boiling for 5 min. After incubation in ice-water for 5 min, the cell debris was removed by centrifugation for 20 min (13000 g, 4 °C) and aspartate was quantified as its o-phthalaldehyde derivative by reversed-phase HPLC (Schrumpf et al., 1992). One unit of PCx activity corresponds to 1 μmol aspartate formed per min. Cell mass was determined by measuring OD₅₇₈ (OD₅₇₈ = 1 corresponded to 0.3 mg dry wt ml⁻¹). Treatment of the cells with concentrations of CTAB higher or lower than 0.3 % decreased the specific PCx activity, and cells not treated with CTAB showed no PCx activity.

In the ¹³C-labelling experiments, NaHCO₃ in the PCx reaction mixture was replaced by H¹³CO₃⁻ (99% atom-enrichment; Cambridge Isotope Laboratories), or 70% of the unlabelled pyruvate was replaced with either [2,¹³C]pyruvate or [3-¹³C]pyruvate (each 99% atom-enrichment; Isotech). The aspartate formed in the assay was purified by cation-exchange chromatography on an Ultrapac 11 μ resin column (Pharmacia) using an FPLC system from Sykam as described previously (Peters-Wendisch et al., 1996). Triethylamine (0.2 M) with a pH gradient from 3.2 to 10.5 was used for elution in 1 ml fractions. The fractions containing aspartate were combined, vacuum-dried, resuspended in 0.6 ml D₂O and then subjected to ¹H-NMR spectroscopy. As an internal standard, sodium 3-trimethylsilylethyl-[2,2'-3,3'-D₅]proionate was included at 2 mM in each sample.

**Enzyme assays with cell-free extracts.** C. glutamicum cells were grown in minimal medium to the exponential phase, washed twice in 20 ml 100 mM Tris/HCl buffer, pH 7, and resuspended in 1 ml of the same buffer containing 20% (v/v) glycerol. After disruption of the cells by sonication (Eikmanns et al., 1991) and subsequent centrifugation (13000 g, 30 min, 4 °C), the supernatant was used for the assays. Protein was
measured by the biuret method (Gornall et al., 1949) with bovine serum albumin as standard.

PCx in cell-free extracts of C. glutamicum was assayed at 30 °C by three different methods. The first was that of Payne & Morris (1969) in which the oxaloacetate formed by PCx is converted to citrate by citrate synthase in the presence of acetyl-CoA and 5,5'-dithio-bis-2-nitrobenzoate. The activity in this assay was monitored by following the increase in A412 due to CoA-dependent formation of 5-thio-2-nitrobenzoate. The second method was that of Milred de Forchetti & Cazzulo (1976), in which the oxaloacetate formed by PCx is converted to malate by malate dehydrogenase in the presence of NADH. The decrease of NADH was monitored at 340 nm. The third method was the discontinuous GOT-coupled assay (Fisher & Magasanik, 1984) described above for measuring the PCx activity in permeabilized cells. PCx from bovine liver (Sigma) and cell-free extracts prepared from Bacillus subtilis DB104 were used as positive controls.

PEPCx and PEPCk were assayed photometrically at 30 °C as described previously (Peters-Wendisch et al., 1993).

**NMR spectroscopy and determination of 13C enrichments.** High-resolution 1H-NMR spectra of aspartate were obtained on a Bruker AMX-400 WB spectrometer operating at 400:13 MHz and equipped with a multichannel interface and a 5 mm inverse probe head. 13C enrichments in C-2 and C-3 of aspartate were calculated from NMR spectra with and without 13C decoupling using the parameters and the method described by Peters-Wendisch et al. (1996). 13C enrichments in C-1 and C-4 of aspartate were quantified by spin echo 1H-NMR difference spectroscopy with and without selective 13C inversion also according to the method described previously (Peters-Wendisch et al., 1996).

**Western blot analysis.** Cell-free extracts of C. glutamicum were prepared as described above. Equal amounts of heat-denatured cell extract protein were loaded and separated on an SDS-PAGE (7.5% or 10% acrylamide, w/v; 10 mA for 14 h) and electroblotted at 5 mA cm⁻² for 30 min onto a polyvinylidene difluoride membrane (Millipore) using a Fast Blot 33 semidry transfer cell from Biometra. After blocking of polyvinylidene difluoride membrane (Millipore) using a Fast Western blot analysis.

**RESULTS**

**PCx activity in C. glutamicum**

Cell-free extracts of C. glutamicum grown on complex medium, and on minimal media containing glucose, lactate or acetate as carbon sources, were tested for PCx activity. Using the citrate-synthase-coupled and the malate-dehydrogenase-coupled assays and a variety of assay conditions, no PCx activity could be detected. Using the discontinuous GOT-coupled PCx assay, pyruvate- and ATP-dependent activity was observed; however, this activity decreased from about 40 nmol min⁻¹ (mg protein)⁻¹ at the start of the reaction to about 4 nmol min⁻¹ (mg protein)⁻¹ after 1 min and to <1 nmol min⁻¹ (mg protein)⁻¹ after 2 min. In contrast, PCx activity could easily and reliably be demonstrated in cell-free extracts of B. subtilis with any of the three assays used (data not shown).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Percentage 13C enrichments in aspartate*</th>
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<tbody>
<tr>
<td>HCO₃⁻ + [2-13C]pyruvate (70%)</td>
<td>1-1 70% 1-1 1-1</td>
</tr>
<tr>
<td>HCO₃⁻ + [3-13C]pyruvate (70%)</td>
<td>1-1 1-1 68% 1-1</td>
</tr>
<tr>
<td>H¹³CO₃⁻ (99%) + pyruvate</td>
<td>1-1 1-1 1-1 98%</td>
</tr>
</tbody>
</table>

*Values are means of two determinations. Absolute errors are <0.5%.

In a further attempt to reliably demonstrate PCx activity in C. glutamicum, we tested permeabilized cells. For this purpose, C. glutamicum WT cells were suspended in HEPES buffer containing 0.3% CTAB and then subjected to the GOT-coupled PCx assay. C. glutamicum cells grown on minimal medium containing lactate as carbon source displayed specific aspartate-forming activity of 45±5 nmol min⁻¹ (mg dry wt)⁻¹. That the observed reaction represented PCx activity was indicated by its linear dependence on cell concentration (up to 2 mg dry wt per assay), incubation time (up to 5 min), and the presence of Mg²⁺ and the substrates pyruvate, ATP and HCO₃⁻. The activity in either HEPES, Tris/HCl, MES or MOPS buffer was approximately the same and optimal over a pH range of 7.0 to 7.5. The reaction rates at different pyruvate concentrations followed Michaelis– Menten kinetics with a Kₘ value of 1.3 mM and a Vₘₐₓ of 50 nmol min⁻¹ (mg dry wt)⁻¹. The Kₘ value for ATP was 0.2 mM. ADP, AMP and ITP inhibited the PCx activity with inhibition constants of 2.6 mM, 0.75 mM and 15 mM, respectively. Additionally, the C. glutamicum PCx activity was efficiently inhibited by acetyl-CoA with a Kᵢ value of 110 μM.

To prove that the aspartate formed by the permeabilized cells originates from pyruvate and HCO₃⁻, i.e. from carboxylation of the methyl group of pyruvate, we performed the GOT-coupled assay in the presence of either [2-13C]pyruvate plus unlabelled HCO₃⁻, [3-13C]pyruvate plus unlabelled HCO₃⁻ or unlabelled pyruvate plus H¹³CO₃⁻ and analysed the purified aspartate by 1H-NMR. As shown in Table 1, the assays with 70% enriched [2-13C]pyruvate or [3-13C]pyruvate resulted in about 70% 13C enrichments in aspartate C-2 and C-3, respectively, and the assay with 99% H¹³CO₃⁻ resulted in 98% 13C enrichment in the C-4 carboxyl group of aspartate. This labelling pattern was expected in the case of direct carboxylation of pyruvate to oxaloacetate and subsequent conversion to aspartate by GOT. Thus, we conclude that aspartate formation in permeabilized C. glutamicum cells was due to PCx activity.

To test whether PCx in C. glutamicum is regulated by
the carbon source, the effect of different growth substrates on the specific activity of the enzyme was determined. The highest activity [45 ± 5 nmol min⁻¹ (mg dry wt)⁻¹] was found in cells grown on lactate or pyruvate; it was significantly lower when the cells were grown on glucose [16 ± 4 nmol min⁻¹ (mg dry wt)⁻¹] or acetate [19 ± 4 nmol min⁻¹ (mg dry wt)⁻¹]. These results suggest that C. glutamicum PCx is regulated by the carbon source, and that the enzyme is more important for C. glutamicum during growth on lactate or pyruvate than on the other two substrates.

The PCx activity was also determined with cells of the PEPCx-negative mutant C. glutamicum WT-PP and of the L-lysine producer MH20-22B. Both strains showed the same specific PCx activities as C. glutamicum WT.

**Effect of biotin on the growth of C. glutamicum**

All PCx enzymes examined so far contain covalently bound biotin as a prosthetic group (Scrutton & Young, 1972). To study whether biotin has an effect on the growth of C. glutamicum, especially on that of the PEPCx-negative mutant WT-PP, we compared growth under biotin limitation and excess. As shown in Fig. 1(a, b), growth of the mutant strain on glucose was considerably more dependent on biotin (optimal at > 50 µg biotin l⁻¹) than growth of the WT (optimal at > 5 µg biotin l⁻¹). These results show that for growth on glucose the demand of C. glutamicum for biotin is significantly higher in the absence of PEPCx than in its presence. In contrast, with lactate as carbon source, the WT and the PEPCx-negative mutant showed about the same high demand for biotin (e.g. in Fig. 1c, d).

However, when fumarate at 35 mM was added to the medium with growth-limiting concentrations of biotin, growth of both strains was completely restored (Fig. 1c, d). Since neither strain grew on fumarate as the only carbon source, these results indicate that a biotin-dependent reaction or pathway in C. glutamicum can be circumvented by supplying the cells with the TCA cycle intermediate fumarate. For optimal growth on acetate as carbon source, both the WT and the PEPCx-negative strain required less than 5 µg biotin l⁻¹ (data not shown). Assuming that the C. glutamicum PCx is a biotin enzyme, these results indicate that (i) PCx is essential as an anaplerotic enzyme for growth on glucose in the absence of PEPCx, (ii) PCx is essential as an anaplerotic enzyme for growth on lactate even in the presence of PEPCx, and (iii) PCx has no anaplerotic function during growth on acetate.
Effect of biotin on the PCx activity of *C. glutamicum*

To investigate whether there is a correlation between the biotin concentration in the growth medium and the PCx activity of *C. glutamicum*, we cultured WT cells on glucose medium supplemented with 100 µg biotin l⁻¹ or 5 µg biotin l⁻¹, and determined their specific PCx activity. The cells showed approximately the same growth on both media; however, the PCx activity of cells growing on the medium with 5 µg biotin l⁻¹ was significantly lower than that of the cells growing in the presence of 100 µg biotin l⁻¹ (Fig. 2a). The same experiments were performed on media containing lactate as the carbon source (Fig. 2b). Under biotin excess, i.e. when the growth of *C. glutamicum* was optimal, the specific PCx activity was relatively high. In contrast, under biotin limitation, i.e. when the growth of *C. glutamicum* was severely impaired, the specific PCx activity was drastically reduced. These data support the idea that PCx is a biotin protein and corroborate the conclusion that PCx is not essential as an anaplerotic enzyme for growth of *C. glutamicum* WT on glucose, but is essential for growth on lactate.

**Identification of biotinylated proteins in *C. glutamicum***

Cell-free extracts of *C. glutamicum* WT and the PEP carboxylase-negative mutant WT-PP grown on glucose medium were assayed for biotinylated proteins by SDS-PAGE and Western blot analysis. Two biotin-containing proteins, with approximate molecular masses of 65 kDa and 125 kDa, were detected in both strains (Fig. 3a). Since the smaller protein represents the biotinylated subunit of an acyl-CoA carboxylase (Jäger et al., 1996) the 125 kDa signal might correspond to the PCx.

To investigate whether there is a relation between
bacterial growth, PCx activity and the 125 kDa biotin protein, we tested C. glutamicum WT cells grown on lactate medium containing 100 µg biotin l⁻¹ or 5 µg biotin l⁻¹ for their content of the 125 kDa biotin protein. Under biotin excess, when the growth of C. glutamicum was optimal and the specific PCx activity was high (see Fig. 2b), significant amounts of the 125 kDa biotin protein were present (Fig. 3b, lanes 1–3). In contrast, under biotin limitation, i.e. when the growth of C. glutamicum was severely impaired and PCx activity was almost not detectable, the 125 kDa biotin protein was essentially absent (Fig. 3b, lanes 4–6). When the same experiments were performed with cells grown on media containing glucose, the 125 kDa biotin protein was again present in the biotin-supplemented cells and not detectable in the biotin-limited cells. These results indicate that the biotinylated 125 kDa protein of C. glutamicum probably represents the PCx enzyme.

**Isolation and characterization of PCx-negative C. glutamicum mutants**

To isolate PCx-negative mutants of C. glutamicum, mutagenized cells of the restriction-deficient strain R127 were plated on complex medium and about 40000 clones were screened for their ability to grow on solid medium with glucose or acetate but not lactate or pyruvate as carbon source. After retesting twice, 15 clones showing this phenotype were obtained. Permeabilized cells of these mutants were then tested for their specific PCx activity. When compared to the parental strain R127, 13 of the 15 showed between 50% and 100% PCx activity [i.e. 7–5–14 nmol min⁻¹ (mg dry wt)⁻¹]. One mutant, designated R127-SP078, showed approximately 10% PCx activity and one, designated R127-SP733, was devoid of any PCx activity (Table 2). These two strains were further tested for PEPCx and PEPCk activities and for their growth phenotype in liquid medium. Cell-free extracts of both mutants showed about the same levels of PEPCx and PEPCk as the parental strain (Table 2) and both mutants grew well on liquid medium with glucose or acetate but did not grow with lactate or pyruvate as carbon source. However, when lactate or pyruvate medium was supplemented with 35 mM fumarate, both mutants showed the same growth as the parental strain in the absence of fumarate. These results suggested that the two isolates are not able to replenish the TCA cycle from lactate and pyruvate and thus, that the phenotype of strains R127-SP733 and R127-SP078 is in fact due to the lack or drastic decrease in anaplerotic PCx activity.

**DISCUSSION**

Previous studies have shown that PEPCx is not essential as an anaplerotic enzyme for C. glutamicum (Peters-Wendisch et al., 1993; Gubler et al., 1994), and from ¹³C-labelling experiments and NMR analysis it was concluded that C. glutamicum must possess alternative anaplerotic activity that carboxylates either PEP or pyruvate (Peters-Wendisch et al., 1996). This conclusion has now been confirmed. The data reported here for the first time provide evidence that C. glutamicum possesses PCx as well as PEPCx, and they suggest that PCx functions exclusively as an anaplerotic enzyme during growth on lactate and pyruvate. In the PEPCx-negative mutant, PCx is obviously also responsible for the net synthesis of oxaloacetate during growth on glucose, and it might even be possible that PCx is the predominant anaplerotic enzyme in C. glutamicum WT during growth on glucose under biotin excess.

In having both PEPCx and PCx, C. glutamicum differs from *Escherichia coli*, *B. subtilis* and *B. stearothermophilus*. In *E. coli*, PEPCx represents the only anaplerotic enzyme during growth on glucose since a defined PEPCx-negative mutant is not able to grow on glucose as the sole carbon source and requires succinate as a supplement (Chao & Liao, 1993). In *B. subtilis* and *B. stearothermophilus*, only PCx is responsible for the net formation of oxaloacetate. Mutants blocked in this enzyme were also unable to grow on minimal glucose or lactate media unless supplemented with TCA cycle intermediates (Diesterhaft & Freese, 1973; Sundaram, 1973). Some bacteria, for example *Pseudomonas citronellolis*, *P. fluorescens*, *Azotobacter vinelandii* and *Thiobacillus novellus*, possess both PEPCx and PCx (O'Brien et al., 1977; Scrutton & Taylor, 1974; Milrad de Forchetti & Cazzulo, 1976; Charles & Willer, 1984). However, to our knowledge it has not been determined whether in these bacteria one or the other of the two enzymes is essential under any given growth condition. Previously, the presence of PCx has been reported in cell-free extracts of *C. glutamicum* subsp. *lactofermentum* (Tosaka et al., 1979), and there has been recent speculation that this enzyme might function in other *C. glutamicum* strains as well (Cocaign-Bousquet et al., 1996). However, in spite of intensive efforts, we and several other groups were unable to reliably measure PCx activity in cell-free extracts of this or any other *C. glutamicum* strain tested (Peters-Wendisch et al., 1993; Jetten et al., 1994; Gubler et al., 1994; Cocaign-Bousquet & Lindley, 1995; Cocaign-Bousquet et al., 1996).

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**Table 2. Specific activities of pyruvate carboxylase (PCx), PEP carboxylase (PEPCx) and PEP carboxykinase (PEPCk) in C. glutamicum R127, R127-SP078 and R127-SP733**

<table>
<thead>
<tr>
<th>Strain</th>
<th>PCx [nmol min⁻¹ (mg dry wt)⁻¹]</th>
<th>PEPCx [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>PEPCk [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>R127</td>
<td>15</td>
<td>231</td>
<td>165</td>
</tr>
<tr>
<td>R127-SP078</td>
<td>1.6</td>
<td>256</td>
<td>159</td>
</tr>
<tr>
<td>R127-SP733</td>
<td>&lt;0.4</td>
<td>262</td>
<td>175</td>
</tr>
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</table>
1996). This surprising finding could be due to instability of the enzyme under the chosen conditions, to loss of an enzyme component, or to loss of an activator molecule during preparation of the extract. PCx from some organisms, for example *Arthrobacter globiformis*, *Aspergillus niger*, chicken and rat liver, is very unstable under certain conditions (at 0°C, pH values > 8 or at low salt concentrations; in the case of *A. globiformis* due to structural changes in the native enzyme) (Gurr & Jones, 1977; Feir & Suzuki, 1969). However, the enzyme from other organisms is stable and easily measured (see, for example, Cazzulo et al., 1970; Scrutton & Young, 1972; Scrutton & Taylor, 1974; Modak & Kelly, 1995). Biochemical and genetic approaches are now needed to find why PCx activity is difficult to measure in cell-free extracts of *C. glutamicum*.

The specific PCx activity in permeabilized cells of *C. glutamicum* depended on the growth medium and varied between 14–19 nmol min⁻¹ (mg dry wt)⁻¹ during growth on acetate or glucose and about 45 nmol min⁻¹ (mg dry wt)⁻¹ during growth on lactate or pyruvate. Assuming that approximately 50% of the dry weight consists of protein, these activities correspond to about 30 and 90 nmol min⁻¹ (mg protein)⁻¹, respectively, and thus are comparable to those observed in cell-free extracts of other organisms such as *A. globiformis* [90 nmol min⁻¹ (mg protein)⁻¹], *Rhizobium etli* [50 nmol min⁻¹ (mg protein)⁻¹], *T. novellus* [25–100 nmol min⁻¹ (mg protein)⁻¹] or *Rhodobacter capsulatus* [20–66 nmol min⁻¹ (mg protein)⁻¹] (Gurr & Jones, 1977; Encarnacion et al., 1995; Charles & Willer, 1984; Willison, 1988). As in *C. glutamicum*, the specific PCx activity of some other organisms, for example *A. vinelandii*, *R. sphaeroides*, *R. capsulatus* and *Saccharomyces cerevisiae*, was three- to fourfold higher when the cells were grown on lactate instead of substrates such as glucose, glycerol, sucrose or malate (Scrutton & Taylor, 1974; Payne & Morris, 1969; Willison, 1988; Young et al., 1969) suggesting a pivotal anaplerotic role of PCx during growth on lactate. For *C. glutamicum*, this suggestion was substantiated by isolating a PCx-negative mutant due to its inability to grow on lactate (and pyruvate).

In its absolute requirement for Mg²⁺, its apparent Kᵥ values of 1·3 mM for pyruvate and 0·2 mM for ATP and its sensitivity to AMP and ADP, the *C. glutamicum* PCx is similar to the PCx enzymes from other organisms (Milrad de Forcetti & Cazzulo, 1976; Scrutton & Taylor, 1974; Cazzulo et al., 1970; Gurr & Jones, 1977; Modak & Kelly, 1995). However, in contrast to all PCx enzymes studied so far, the *C. glutamicum* enzyme was inhibited by acetyl-CoA. Due to this feature, the *C. glutamicum* PCx does not conform to the classification of PCx enzymes by Scrutton & Young (1972), which is based on the degree of activation of the enzyme by acetyl-CoA. Some PCx enzymes, for example those from *B. subtilis*, *B. stearothermophilus* and *R. capsulatus*, are strictly dependent on the presence of acetyl-CoA (Cazzulo et al., 1970; Diesterhaft & Freese, 1973; Modak & Kelly, 1995). The PCx enzymes of *A. globiformis*, *T. novellus* and *S. cerevisiae* are active without acetyl-CoA but they are stimulated significantly by it (Gurr & Jones, 1977; Charles & Willer, 1984; Ruiz-Amil et al., 1965), whereas those of *P. fluorescens*, *P. citronellolis*, *A. vinelandii* and *A. niger* are completely independent of acetyl-CoA (Milrad de Forcetti & Cazzulo, 1976; Seubert & Weicker, 1969; Scrutton & Taylor, 1974; Feir & Suzuki, 1969). The effective inhibition of the *C. glutamicum* PCx by acetyl-CoA together with the strong inhibition of the *C. glutamicum* PEPCx by aspartate (Eikmanns et al., 1989) might ensure that during growth of *C. glutamicum* on acetate or other precursors of acetyl-CoA the anaplerotic carboxylation reactions do not interfere with gluconeogenesis, i.e. with PEP and/or pyruvate formation from oxaloacetate by PEPCx and/or oxaloacetate decarboxylase. Both these enzymes have been found in *C. glutamicum* and shown to be formed under glycolytic as well as under gluconeogenic conditions (Jetten & Sinskey, 1993; Peters-Wendisch et al., 1993; Jetten & Sinskey, 1995; Jetten et al., 1994). Since PEPCx and PCx are also formed under both conditions, a tight effector-modulated regulation of all the enzymes involved in interconverting PEP, pyruvate and oxaloacetate is required to properly adjust the carbon flux at this branch point within central metabolism, and to prevent extensive futile cycling. However, while the regulatory properties of the *C. glutamicum* PEPCx, PEPCx and oxaloacetate decarboxylase are well known [PEPCx is inhibited by aspartate and activated by acetyl-CoA and fructose-1,6-bisphosphate (Mori & Shioh, 1985a, b; Eikmanns et al., 1989); PEPCx is inhibited by ADP and ATP (Jetten & Sinskey, 1993); oxaloacetate decarboxylase is inhibited by ADP, CoA and succinate (Jetten & Sinskey, 1995)], there are still some uncertainties about the regulation of PCx, since, for example, the effects of aspartate and 2-oxoglutarate, which both are potent inhibitors of PCx enzymes from some other organisms (e.g. Gurr & Jones, 1977; Modak & Kelly, 1995), could not be tested due to the principle of the GOT-coupled assay applied in this work.

Besides PEPCx, PEPCx, PCx and oxaloacetate decarboxylase, the decarboxylating/carboxylating enzymes located at the metabolic node around pyruvate in *C. glutamicum* include the NADP-dependent malic enzyme (Cocaïgn-Bousquet & Lindley, 1995; Vallino & Stephanopoulos, 1993). In principle, the reaction catalysed by this enzyme is reversible, but in most organisms the malic enzyme is assumed to catalyse the oxidative decarboxylation of malate rather than the reductive carboxylation of pyruvate (Kornberg, 1966). Although the role of the malic enzyme in *C. glutamicum* has never been clearly defined, Cocaïgn-Bousquet & Lindley (1995) deduced from kinetic data of steady-state chemostat and batch cultures growing on lactate that the enzyme is involved in a metabolic cycle generating NADPH from NADH and thus provides reducing equivalents for anabolic metabolism. In this cycle, malate is decarboxylated to pyruvate by the NADP-dependent malic enzyme, pyruvate is carboxylated to
oxaloacetate by PCx, and oxaloacetate is then reduced to malate by NADH-dependent malate dehydrogenase. Our results indicating that PCx is exclusively responsible for the net synthesis of oxaloacetate in *C. glutamicum* WT during growth on lactate and in the PEPCx-negative mutant during growth on glucose agree with this model. They indicate that the malic enzyme does not catalyze the reductive carboxylation of pyruvate, and thus does not have an anaplerotic function under the conditions employed in this study.

It is evident that there is still a lot to be learned about PCx, the anaplerotic carboxylation and the gluconeogenic and/or NADPH-generating decarboxylation of pyruvate, and thus does not have an anaplerotic function under the conditions employed in this study.

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### REFERENCES


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