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ORIGINAL PAPER

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# Glutamate as an inhibitor of phosphoenolpyruvate carboxylase activity in *Corynebacterium glutamicum*

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**Abstract** The glutamate-producing bacterium, *Corynebacterium glutamicum* is known to possess two anaplerotic enzymes: pyruvate carboxylase (Pc) and phosphoenolpyruvate carboxylase (PEPc). In vitro, this latter enzyme appeared to be inhibited by different glutamic acid salts, whereas ammonium-glutamate had no influence on Pc activity. To investigate the in vivo relevance of PEPc activity inhibition, the intracellular concentration of glutamate was determined throughout the glutamate-producing process. The intracellular concentration was then shown to be sufficient to induce a dramatic inhibition of PEPc activity during the process. As a consequence, intracellular accumulation of glutamate could be at least partially responsible for the weak participation of PEPc within the anaplerosis activity in amino-acid-producing strains of *C. glutamicum*.

**Keywords** Phosphoenolpyruvate carboxylase · Pyruvate carboxylase glutamic acid fermentation · *Corynebacterium glutamicum* · Regulation

## Introduction

*Corynebacterium glutamicum* is widely used for the production of amino acids such as glutamate and lysine [6]. Because of their strong potential as industrial microorganisms, corynebacteria have been extensively investigated over the past 30 years. Our knowledge of their metabolism and its regulation has improved greatly and will presumably increase rapidly with the recent sequencing of the *C. glutamicum* genome [9]. So far, classical selection has succeeded in creating strains able to produce large amounts of glutamate. The current challenge is to further increase glutamate yield via metabolic engineering. For this purpose, metabolic steps potentially restraining the channelling of carbon flow from glucose to glutamate have to be identified. Particular attention has been paid to anaplerotic pathways.

In *C. glutamicum*, two anaplerotic enzymes are responsible for TCA cycle replenishment via oxaloacetate during growth on carbohydrates: phosphoenolpyruvate carboxylase (PEPc) and pyruvate carboxylase (Pc) [23]. Several approaches were used to unravel the respective roles of these two enzymes during amino acid production. The metabolic consequences of Pc overexpression depended on the genetic background of the strain, resulting in either no modification of lysine production (*C. glutamicum* ATCC 21253 and ATCC 21799 [11]) or an improvement in both glutamate and lysine productions (strains related to *C. glutamicum* ATCC 13032 [24]). It was also shown that, in the presence of active Pc, amplification of PEPc activity had no favourable influence on glutamate synthesis in *C. glutamicum* 2262 [3]. Further studies using $^{13}$C-nuclear magnetic resonance spectroscopy [20, 21] confirmed that PEPc only marginally contributed to anaplerosis, Pc playing the major role as the anaplerotic enzyme in *C. glutamicum*. However, thus far the in vivo regulation responsible for this carbon partitioning between PEPc and Pc remains unclear.

In *Brevibacterium flavum*, PEPc was shown to be activated by metallic cations ($Mn^{2+}$ and $Mg^{2+}$) or intermediates such as acetyl-CoA and fructose 1, 6 bi-phosphate [16, 18]. However, no synergistic activation was observed between acetyl-CoA and fructose 1, 6 bi-phosphate as previously observed in *Escherichia coli*

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[8, 18]. Conversely, PEPc from corynebacteria is inhibited by pyruvate, acetate and TCA cycle intermediates (α-ketoglutarate, citrate, isocitrate, succinate, fumarate and malate). Some amino-acids (aspartate, threonine) also have an inhibitory effect on PEPc; however, 50 mM glutamate was shown to have very little influence on *B. flavum* PEPc activity [17]. Furthermore, a synergistic inhibition was measured when aspartate and α-ketoglutarate were combined [17, 19].

The gene encoding Pc has only recently been isolated in *C. glutamicum* [10, 23] and little is known about the regulation of the activity of this enzyme in corynebacteria, although it was shown to be inhibited by acetyl CoA, ADP, AMP, ITP [22] and aspartate [11].

In order to see if yet unknown effectors of PEPc and Pc activities could explain the in vivo repartition of carbon flow through these two enzymes, we investigated the sensitivity of PEPc and Pc to glutamic acid salts. For this purpose PEPc and Pc activities from a glutamate-producing corynebacterium, *C. glutamicum* 2262 were assayed in vitro with various glutamic acid salts. In order to estimate the physiological relevance for in vivo enzyme activities of these in vitro assays, the intracellular concentration of glutamate during the glutamic acid production process was measured.

## Materials and methods

### Bacterial strain and medium composition

The glutamate producer strain used throughout this study was *C. glutamicum* 2262 [2]. The composition of the medium used was based on MCGC medium [26] although citrate (used as chelating agent) was replaced by 3 mg/l deferoxamine.

### Culture conditions

Batch cultures (for enzyme activity determinations) and seeds were performed in 500 ml baffled flasks, at 33°C, in modified MCGC medium containing 34 g/l glucose (PEPc determination) or lactate (Pc determination) and supplemented with 12 g/l $Na_2HPO_4$ and 1.9 g/l urea. Flasks were shaken at 200 rpm. The pH was initially adjusted to 7.6 with 12 *N* NaOH. For fed-batch cultures, a process similar to the industrial culture, yielding high glutamate production by increasing the culture temperature from 33 to 39°C, was followed [2].

### Enzyme assays

PEPc activity determination was performed on crude extracts obtained by sonication of the corynebacteria on ice. Cells were harvested and washed twice with 0.2% (w/v) KCl and resuspended in Tris-tricarballylate buffer (200 mM) containing $MgCl_2$ (5 mM) and glycerol (20%, v/v). Eight sonications (at 200 W), lasting 30 s each, were performed. Two consecutive sonications were separated by 1 min. PEPc was assayed according to a protocol based on the method described by Mori and Shiio [16]. Endogenous NADH consumption was taken into account in order to avoid overestimation of PEPc activity. Briefly, OD (340 nm) was measured without substrate (PEP) addition to determine endogenous oxidation of NADH; PEPc activity was then initiated by addition of PEP. PEPc activity was calculated as the difference between the rate of NADH oxidation before and after PEP addition. The accuracy of the method was evaluated on ten independent assays. With the PEPc activities determined, the confidence interval ($P < 0.05$) was ±20%.

Pc activity was measured in permeabilised cells as previously described by Uy and co-workers; the accuracy of the method was ±15% [25]. Protein concentration was determined using Bio-Rad kit no. 500-0001 with bovine gamma globulin as protein standard (Bio-Rad, Hercules, Calif.). All results reported are averages of triplicate assays.

### Determination of intracellular amino acid concentration

Cells of a 500 μl sample were separated from the medium by centrifugation on 500 μl of a mixture of 42.5% 1-bromo-hexadecane and 57.5% 1-bromo-heptane as separation layer and 250 μl 21% $HClO_4$ as acid fixation layer: 64,000 *g*, 4 min and 4°C. The upper layers containing the extracellular medium and the bromo-alcans were removed before freezing the samples (−20°C). The sedimented cells in the acid layer were disrupted using three cycles of freezing/thawing. The resulting extracts were neutralised by adding 5 M NaOH. These extracts were centrifuged at 4°C, 32,000 *g*, 10 min and the supernatants were used for amino acid detection, whereas cell fragments, denatured proteins, and residual bromo-alcans were removed in the sediment. Amino acids were quantified as their ortho-phtaldialdehyde derivatives by automatic precolumn derivatisation and separation on a Hewlett-Packard C18 column using a model LC 1090 ChemStation [13].

Prior to the determination of internal concentrations of glutamate, the diffusion of hydrophilic compounds such as glutamate through the bromo-alcan layer was estimated. For this purpose, increasing concentrations of sodium thiosulfate, previously described as non-penetrating in cells [14], were added to some crude extracts. The percentage of contamination was then calculated in accordance with the external concentrations of sodium thiosulfate and of biomass. As expected, contamination increased with the external concentration of the hydrophilic compound and with the biomass concentration. For the internal glutamate values of the fed-batch culture presented in this work, this estimated contamination was subtracted from the raw measurement. However, when the external glutamate concentration

exceeded 50 g/l, the contamination was too high to accurately measure the internal glutamate concentration [12].

Cellular volume measurement

The cellular volume was determined during the growth phase of the glutamic acid fermentation (just before the temperature shift) and after the temperature increase until the end of the culture. The volume measurement was performed using a CASY analyser device (Schärfe System, Reutlingen, Germany). Collected cells were diluted in an isotonic buffer according to the manufacturer's protocol and aspirated through a precision measuring capillary. While passing through the measuring capillary, in a low voltage field between two platinum electrodes, cells yield electrical signals of different amplitude, pulse width, time-course, and resulting pulse area. The analysed pulse areas of cell signals are cumulated and assigned in a calibrated multi-channel analyser that has 512,000 differentiated size channels. To estimate the volumetric intracellular concentration of glutamate, the cellular volume measured was extrapolated as an intracellular water volume.

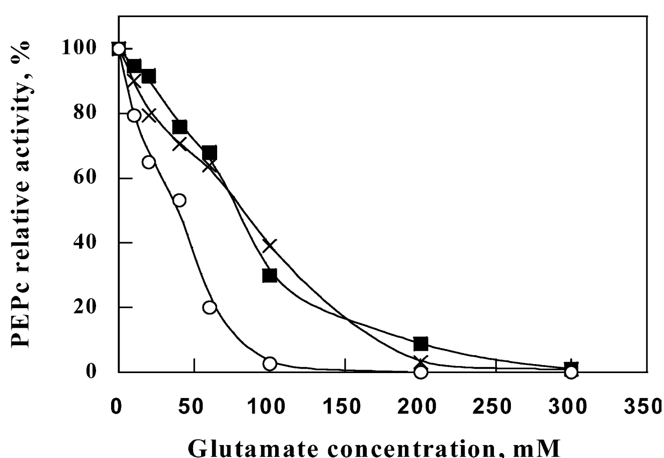


**Fig. 1** Inhibition of phosphoenolpyruvate carboxylase (PEPc) activity from *C. glutamicum* 2262 in the presence of increasing concentrations of sodium- (*crosses*), potassium- (*filled squares*) or ammonium- (*open circles*) glutamate

## Results

Influence of glutamic acid salts on PEPc and Pc activities

Sodium-, potassium- or ammonium-glutamate (0–300 mM) were added to PEPc in vitro assays in order to investigate the influence of glutamic acid salts on PEPc activity (Fig. 1). Contrary to Mori and Shiio [15], who reported a weak (2–5%) inhibition in *B. flavum* with a similar range of glutamate concentrations, PEPc activity in *C. glutamicum* 2262 was severely reduced with as little as 50 mM glutamic acid salt (25% decrease). The activity decreased dramatically at glutamate concentrations higher than 60 mM, and was totally inhibited by concentrations above 300 mM. While sodium and potassium salts resulted in a similar degree of inhibition, ammonium-glutamate addition caused a significantly stronger inhibition; 100 mM of this glutamic acid salt nearly abolished PEPc activity. To identify and quantify the effect of cations on PEPc activity, coryneform cell lysates were incubated in the presence of NaCl, KCl or $NH_4Cl$. While no inhibition was observed when 300 mM NaCl or KCl were added to the assay (data not shown), as little as 20 mM $NH_4Cl$ caused an immediate decrease in PEPc activity, and the addition of 300 mM $NH_4Cl$ resulted in 44% inhibition (Table 1). To investigate if ammonium ions and glutamic acid were synergistic effectors of PEPc, we quantified the individual inhibition by glutamic acid or ammonium ions in vitro. The inhibition measured in the presence of ammonium-glutamate was always higher than the cumulative inhibition calculated from individual inhibitions by ammonium ions or glutamic acid (Table 1). This result suggested that these two molecules might synergistically inhibit PEPc activity. It should be borne in mind that, during the glutamate-producing process, the pH is regulated by ammonia addition. The extracellular ammonia concentration increased throughout the culture and

**Table 1** Inhibition of phosphoenolpyruvate carboxylase (PEPc) from *Corynebacterium glutamicum* 2262 in the presence of ammonium chloride, sodium glutamate or ammonium glutamate. Comparison with the calculated inhibition of either ammonium ions or glutamic acid revealed cumulative inhibition of PEPc

| Concentration (mM) | Inhibition (%) | | | Calculated cumulative inhibition[a] |
|---|---|---|---|---|
| | $NH_4Cl$ | Na-glutamate | $NH_4$-glutamate | |
| 0 | 0 | 0 | 0 | 0 |
| 20 | 12 | 21 | 35 | 30 |
| 40 | 17 | 30 | 47 | 42 |
| 60 | 26 | 36 | 80 | 53 |
| 100 | 30 | 61 | 97 | 73 |
| 200 | 43 | 97 | 100 | 98 |
| 300 | 44 | 99 | 100 | 99 |

[a]Cumulative inhibition was calculated as follows: Inhibition (%) = $\left[1 - \left(1 - \frac{\%\text{ inhibition } NH_4^+}{100}\right) \times \left(1 - \frac{\%\text{ inhibition glutamate}}{100}\right)\right] \times 100$

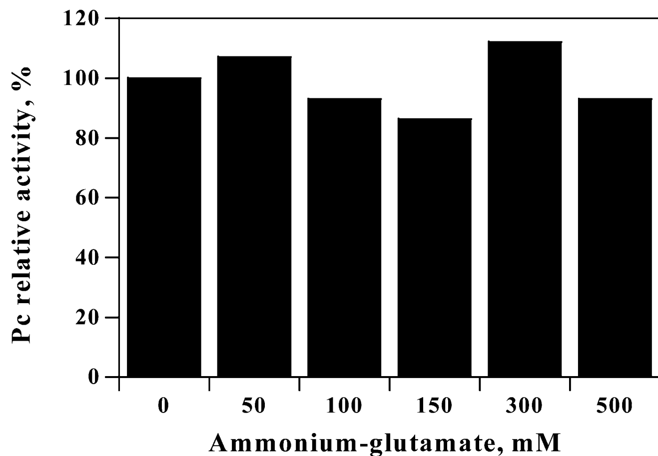


**Fig. 2** Inhibition of pyruvate carboxylase (Pc) activity from *Corynebacterium glutamicum* 2262 in the presence of increasing concentrations of ammonium-glutamate

reached about 700 mM after a 24 h period [1]. It is therefore reasonable to assume that, intracellularly, ammonium-glutamate represents the main glutamic acid salt. Ammonium-glutamate added to the in vitro Pc assay at concentrations ranging from 0 to 500 mM did not affect Pc activity (Fig. 2).

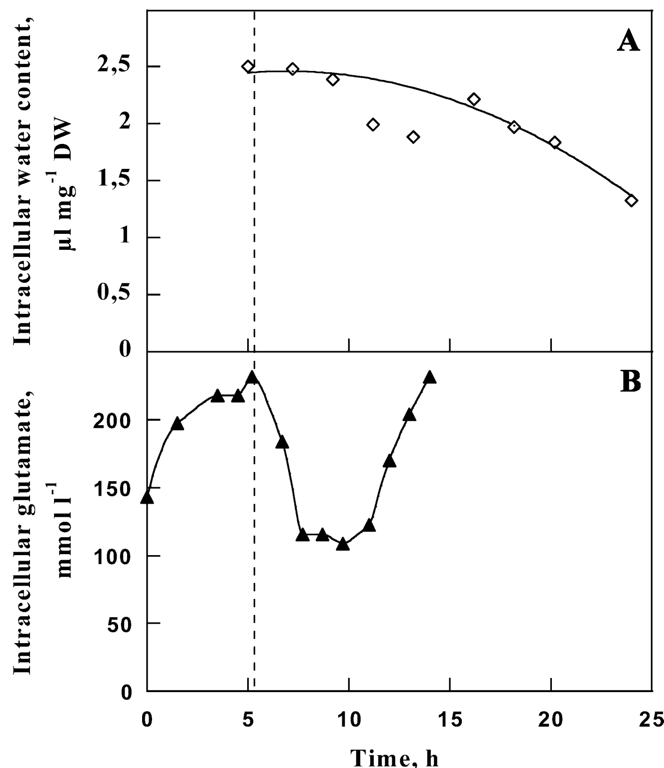


**Fig. 3** Intracellular water content (**a**) and intracellular glutamate concentration (**b**) during temperature-triggered glutamic acid fermentation using *C. glutamicum* 2262. *Vertical dashed line* Time at which the temperature of the culture medium was increased from 33 to 39°C

### Determination of intracellular glutamate concentration during the process of glutamate production

To investigate the in vivo relevance of PEPc activity inhibition by glutamate, determination of the intracellular concentration of this amino acid and its evolution during glutamic acid fermentation was essential (Fig. 3). In many earlier studies, intracellular concentrations were expressed as moles or grams per cell dry weight (DW). The use of these measurements is based on the assumption that the cellular volume of the bacteria remains unchanged. However it has been previously shown that the volume of *C. glutamicum* decreases during the glutamate production phase [4, 7, 15], probably as a consequence of the increasing osmotic pressure. In order to express the intracellular glutamate concentration in grams or moles per litre, we quantified the water content of *C. glutamicum* 2262 during temperature-triggered glutamic acid fermentation. The cells' water content remained constant (2.5 µl/mg DW) up to 3 h after the onset of glutamate excretion (Fig. 3a). A decrease was then measured until the end of the culture (down to 1.4 µl/mg DW). Using this water content data, the intracellular glutamate concentration was expressed as a millimolar concentration and was calculated throughout the fermentation (Fig. 3b). During the growth phase, the intracellular glutamate reached a steady concentration of 215 mM (32 g/l). However, the temperature increase from 33 to 39°C used to trigger glutamate excretion resulted in a significant decrease in the intracellular glutamate concentration. After 2 h of fast decrease, this concentration stabilised around 110 mM (16 g/l) for 3 h. Thereafter, the internal glutamate content rose again and reached 240 mM (35 g l$^{-1}$) 14 h after the start of the culture (i.e. 8.5 h after the shift to glutamate production). Further glutamate measurements are not representative of the internal concentration due to contamination by the high extracellular glutamate titre above 50 g/l.

## Discussion

For the first time, the inhibition of PEPc activity by glutamic acid salts was clearly demonstrated in a glutamate-producing strain of *C. glutamicum*. This inhibition is of in vivo relevance since the intracellular accumulation of glutamate during glutamic acid fermentation (between 110 and 240 mM) could be sufficient to induce strong, if not complete, inhibition of PEPc activity. We also demonstrated that Pc, the other anaplerotic enzyme in corynebacteria, is not at all affected by glutamic acid salts. Glutamate could thus play an essential role in controlling carbon flow partitioning at the PEP/pyruvate metabolic node and explain the predominance of Pc as anaplerotic enzyme.

Nevertheless, glutamate is not the only effector of PEPc activity. For instance, acetyl CoA was shown to activate PEPc activity [1, 18] and to inhibit Pc activity [22]. Compared to glutamate, this metabolite might thus have the opposite influence on anaplerotic enzymes. Fructose-1,6-bisphosphate was also previously identified as an activator of PEPc activity [1, 18] and a non negligible level of this metabolite was detected during the temperature-triggered process with *C. glutamicum* 2262 [5]. These metabolites might thus be responsible for at least a partial restoration of PEPc activity despite the inhibition by glutamate.

We previously postulated that PEPc activity could be of importance for glutamate production during the very first hours following the onset of glutamate excretion [3]. During this period, the intracellular concentration of glutamate decreased to 110 mM. Considering glutamate as the sole effector, such an in vivo concentration would still seriously inhibit PEPc (Fig. 1). However, the presence in vivo of some activators, such as those mentioned above, could restore significant PEPc activity. Thus, the decrease in the intracellular glutamate concentration immediately after the temperature up-shock, would be in agreement with a more important anaplerotic role of the PEPc activity at this precise period of the culture.

As a consequence of the data presented in this work, further experiments have to be performed to elucidate the precise regulation of PEPc and Pc activities during glutamic acid fermentation, and then to identify the metabolite(s) responsible for the predominance of Pc among anaplerotic enzymes. Accordingly, exhaustive identification of PEPc and Pc effectors, as well as quantification of their intracellular concentrations during the glutamate-producing process constitute the next step of this work.

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