Glutamate Excretion Mechanism in *Corynebacterium glutamicum*: Triggering by Biotin Starvation or by Surfactant Addition

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Cells of an industrial strain of the L-glutamate producer *Corynebacterium glutamicum* grown in biotin-starvation conditions lost 30% of their membrane phospholipids. This was accompanied by a progressive decrease in the rate of accumulation of glutamate and of the intracellular glutamate accumulated at the plateau. Addition of an acylated surfactant to growing cultures of the same strain also induced a progressive loss of glutamate uptake, without changing the K_T of the process. The surfactant-treated cells almost completely excreted the labelled glutamate loaded during a preincubation. These results, together with those obtained previously, allow us to propose that glutamate excretion is mediated by a glutamate permease, after uncoupling of this uptake system resulting from a marked loss of membrane phospholipids. This loss of phospholipids can be induced either by biotin starvation or by addition of an acylated surfactant to growing cells.

INTRODUCTION

Glutamate excretion by industrial strains of *Corynebacterium glutamicum*, or by related organisms, is triggered in industrial conditions by inducing modifications of the cell membrane (Demain & Birnbaum, 1968). In early industrial processes, fatty acid synthesis was impaired by biotin starvation of biotin-auxotrophic strains (Shiio *et al.*, 1962). Since the 1960s, glutamate excretion has been obtained by adding a surfactant, acylated with saturated fatty acids, to exponentially growing cultures: the exogenous fatty acids are incorporated into the lipids (Takinami *et al.*, 1968), *de-novo* fatty acid synthesis is inhibited and the cells lose nearly half of their membrane lipids (Huchénq *et al.*, 1984).

In a previous paper (Clément *et al.*, 1984) we showed that the acylated surfactant induced selective uncoupling of the glutamate uptake system, and it was postulated that glutamate was excreted via the uncoupled glutamate permease. It is not known if the uncoupling was due to alteration of the permease directly by the added surfactant, or to the observed membrane modifications. We therefore examined the effect of biotin starvation on the membrane lipid content and on glutamate uptake. Parallel experiments were done with surfactant-treated cells to complete our previous results and to compare further the influence of the two excretion-promoting conditions on the cell membrane.

METHODS

Chemicals. ^{14}C -labelled L-glutamate and L-serine were purchased from the Commissariat à l'Energie Atomique (CEA Saclay, France) and ^{32}P phosphate from Amersham.

Polyoxyethyleneglycol stearate (Simulsol M49), subsequently called 'acylated surfactant', was obtained from Seppic-Montanoir (Paris, France).

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

Bacterial growth. The strain of *Corynebacterium glutamicum* used was obtained from a glutamate-producing company, and was described in our previous work (Clément *et al.*, 1984). Cells were grown in Erlenmeyer flasks on a rotary shaker, at 30 °C, final pH 8, in a basal salts medium, containing, per litre: 3 g (NH₄)₂SO₄, 1 g KH₂PO₄, 3 g K₂HPO₄, 0.1 g MgSO₄·7H₂O, 1 mg CaCl₂, 8.5 mg ZnSO₄·7H₂O, 72 mg MnCl₂·4H₂O and 37 mg (NH₄)₂MoO₄. For normal growth the basal medium was supplemented with 30 µg biotin, 100 µg thiamin, 4.5 g urea and 20 g sucrose per litre. For biotin starvation, the same supplements were added, but only 0.3 µg biotin.

The acylated surfactant was added at a concentration of 0.5% (v/v) to cultures with an OD₆₅₀ of about 5 units (as measured with a Zeiss PMQ III spectrophotometer), corresponding to 1 mg cell dry weight ml⁻¹.

Amino acid uptake. Collecting of cells and amino acid uptake procedures have been described (Clément *et al.*, 1984). Three to five uptake experiments were performed for each experimental condition (Figs 2 to 5).

Lipid analysis. Lipids were extracted from sedimented cells or from culture samples and analysed according to our usual procedures (Huchenq *et al.*, 1984). ³²P Incorporation into lipids was followed in five independent experiments with the same result.

RESULTS AND DISCUSSION

Effects of biotin starvation

It was checked that biotin starvation did not significantly change the cell fatty acid composition (not shown). It can be concluded from Fig. 1 (*a*) that once growth has been inhibited by biotin starvation (Fig. 1 *b*), the cells lose about one-third of their phospholipids. As stated in the Introduction, the same phenomenon was observed when an acylated surfactant was added to the culture.

The activities of the glutamate and serine uptake systems were followed for normal and biotin-starved cultures during growth (Fig. 2). The activity of the serine uptake system was studied for reference, because it depends on the protonmotive force for its energy, as does the glutamate uptake system (Clément *et al.*, 1984). As long as the cells were actively growing (Fig. 2 *a, b*), biotin starvation did not alter the activity of the two uptake systems (as seen from amino acid accumulation over 5 min), but a slightly lower accumulation plateau was reached in starved cells. This could be due to a lowering of the protonmotive force. Clearcut differences between the uptake systems of normal and of starved cells appeared for cells harvested at the beginning of the stationary growth phase (Fig. 2 *c, d*). The activity of both uptake systems was lowered by biotin starvation, but whereas there was nearly a 75% decrease in the activity of the glutamate uptake system, there was only a 50% decrease in the activity of the serine uptake system. Differences at the accumulation plateau were even greater. It can be concluded that, as observed for the effect of an acylated surfactant (Clément *et al.*, 1984), the glutamate uptake system was more impaired by the membrane modification than was the serine uptake system.

Effects of an acylated surfactant

The above-noted progressive inhibition of glutamate uptake by biotin starvation raised the possibility of the existence of similar kinetics for the effect of the acylated surfactant. As seen in Fig. 3, the maximum inhibition of glutamate uptake was reached after 30 min growth in the presence of the surfactant. This corresponds to the maximum effect of the surfactant on the cell phospholipid content (Huchenq *et al.*, 1984). In contrast to the effect of biotin starvation, the acylated surfactant did not impair the serine uptake system (Clément *et al.*, 1984), and did not modify the growth curve. The velocity ($V_{\max}$) of the uptake was reduced by the acylated surfactant, but the constant K_T was not changed since the four K_T determinations performed with normal and with starved cells were between 2×10^{-5} and 3×10^{-5} M. Thus, it is likely that the surfactant did not significantly alter the binding site of the substrate on the permease.

To be able to excrete glutamate, the permease has to interact with the glutamate at the inner face of the bacterial membrane. We checked that this was possible by showing that non-treated cells were able to exchange the accumulated amino acid for non-labelled glutamate added in the medium (Fig. 4). The exchange process was even more efficient in decreasing the [¹⁴C]glutamate content of the cells than was the addition of an uncoupler. Such an exchange process has been described for *Escherichia coli* (Cohen & Rickenberg, 1956; Kepes, 1971) for molecules accumulated by the protonmotive force.

Fig. 1. ^{32}P incorporation into lipids of normal and biotin-starved cells. (a) Labelling of lipids extracted from 0.8 ml samples of the cultures. Crude counts were corrected for culture growth by dividing them by the culture OD_{650} and expressed as percentages of the values for the first sample of each of the two cultures (samples taken after 4.5 h growth; crude counts of 130 c.p.m. and 460 c.p.m. were obtained for normal and starved cells, respectively, in the experiment presented). In the experiment presented, there were 0.25×10^6 and 5×10^6 c.p.m. per ml of medium for normal and low biotin cultures, respectively. (b) Growth curves of the cultures. $\blacktriangle$, Normal culture; $\circ$, biotin-starved culture.

Fig. 2. Activity of the L-glutamate and of the L-serine uptake systems in normal and biotin-starved cells. (a) Glutamate uptake of cells harvested during the exponential growth phase; (b) serine uptake of the same cells as in (a). (c) Glutamate uptake of cells harvested during the transition between the exponential and the stationary growth phases; (d) serine uptake of the same cells as in (c). $\blacktriangle$, Control cells; $\square$, biotin-starved cells.

Excretion of accumulated glutamate was then looked for by loading normal or surfactant-treated cells with labelled glutamate, then diluting them in a medium without glutamate (Fig. 5). It appeared that 20 min after the dilution step, surfactant-treated cells retained only 15% of their initial labelled glutamate content. The control, non-treated cells doubled their labelling within 20 min of dilution. This showed that the surfactant-treated cells, but not the control cells, were able to excrete glutamate efficiently.

Conclusion

The above results, together with those obtained previously (Clément *et al.*, 1984) indicate that glutamate excretion is triggered by a large decrease in the phospholipid content of the bacterial membrane observed on either biotin starvation or surfactant treatment of cells. The resulting membrane alterations selectively uncouple the glutamate uptake system from its energy source,

Fig. 3. L-Glutamate uptake by cells grown for different times in the presence of the acylated surfactant. Uptake was followed for control cells (▲) or for cells grown in the presence of the acylated surfactant for 15 min (○), 30 min (□) or 60 min (●).

Fig. 4

Fig. 5

Fig. 4. Exchange ability of the L-glutamate uptake system. Cells were first allowed to accumulate L-[^{14}C]glutamate under standard conditions (see Methods). At the time indicated by the arrow the following additions were made: non-labelled L-glutamate at a final concentration five times higher than the labelled L-glutamate (○); an uncoupler (CCCP, $10\ \mu\text{M}$) (●). ▲, Control.

Fig. 5. L-Glutamate excretion by preloaded cells. Cells were loaded for 30 min with L-[^{14}C]glutamate ($25\ \mu\text{M}$ final concentration), then 0.1 ml of the cell suspension was diluted in 0.9 ml of the transport medium without glutamate. The radioactivity of cells was followed in 0.1 ml samples. The measured values were expressed as percentages of the radioactivity values of samples taken immediately after the dilution step in the non-radioactive medium (time 0). In the experiment presented these 100% values were 36×10^6 and 16×10^6 c.p.m. (mg dry weight) $^{-1}$ for normal cells and surfactant-treated cells, respectively. ▲, Non-treated cells (control); ○, cells grown for 30 min in the presence of the acylated surfactant.

the protonmotive force, but apparently without impairing the ability of the permease to interact with its substrate. Once uncoupled, the glutamate permease carries the substrate according to its electrochemical potential, i.e. by excreting glutamate from actively growing cells.

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