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Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation

Marcella Gutmann, Christian Hoischen and Reinhard Krämer

Institut für Biotechnologie I, Forschungszentrum Jülich, Jülich (Germany)

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Previous studies have demonstrated the involvement of a carrier system in glutamate secretion by *Corynebacterium glutamicum* under biotin limitation (Hoischen, C. and Krämer, R. (1989) Arch. Microbiol. 151, 342–347). In a detailed analysis of the export process we found secretion to be independent of secondary forces: (i) glutamate was secreted at high rate even when external glutamate exceeded the internal concentration, (ii) movement of neither protons nor potassium or chloride ions was found to be coupled to glutamate secretion, and (iii) secretion continued unaffected after breakdown of the membrane potential. Instead, under conditions leading to variation of glutamate secretion activity, a correlation of secretion rate and the intracellular ATP-pool was observed. Thus, ATP or a related high-energy metabolite is thought to be involved in the activity of the glutamate secretion system.

Introduction

In recent years an increasing body of evidence for carrier mediated solute efflux in bacteria has accumulated. The compounds transported comprise organic acids [1,2], carbohydrates [3,4], heavy metal ions [5,6], antibiotics [7], and toxic compounds [8]. A number of different export mechanisms have been identified: The chemical gradient of lactic acid drives lactic acid excretion in symport with protons in *Escherichia coli* and *Lactococcus lactis* [9–11]. Proton motive force driven secondary transport has been identified for export of cationic dyes in *Escherichia coli* [8] and efflux mediated multidrug resistance in *Bacillus subtilis* [12]. ATP serves as source of energy for plasmid-encoded heavy metal exporters [13]. The physiological role of the export carriers mentioned can easily be rationalized. They are involved in osmotic homeostasis, detoxification and disposal of metabolic endproducts in fermentative bacteria. The function of amino acid efflux in bacteria evades such rapid comprehension, as amino acids are valuable nutrients that are taken up by specific carriers in numerous organisms.

Corynebacterium glutamicum is best known for its ability to secrete large amounts of amino acids [14] and is thus used for amino acid production on industrial scale. We study the secretion of isoleucine [15], lysine [16,17] and glutamate [18] by this organism, and we were able to show export carrier systems to be active in all three cases. This is in contradiction to the efflux models generally discussed in literature, proposing efflux to be caused by either passive diffusion across a leaky cell membrane [19,20] or by the respective amino acid uptake system working in inverted direction [21].

Isoleucine and lysine are secreted in *C. glutamicum* by secondary transport, under conditions when a high intracellular pool of the respective amino acid is accumulated [15–17]. Glutamate secretion, on the other hand, is observed in the wild type only after special treatment of the cells: growth under biotin limitation [22], application of penicillin [14] or of certain surfactants [23] all lead to efflux of glutamate from the cells. A recent effort to analyze the mechanism of glutamate secretion has focused on secretion triggered by amine surfactants [24]. The authors conlude that glutamate is secreted under these conditions by a passive process which is triggered by a change of the membrane status of the cells. For biotin-starved cells we have shown previously that glutamate secretion does not occur by passive diffusion but that it is characterized by properties attributable only to a carrier mediated process: secretion in fact is energy dependent and regulated by the metabolic status of the cells [18]. The reduced

Correspondence to: R. Krämer, Institut für Biotechnologie I, Forschungszentrum Jülich, Postfach 1913, Jülich, Germany.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP, tetraphenylphosphonium bromide; dw, dry weight; Mops, 3-(*N*-morpholino)propanesulfonic acid.

phospholipid content of the cytoplasmic membrane due to lack of biotin, although necessary for induction of secretion, does not lead to permeabilization of the cells [25]. The present study deals with mechanism and energetic coupling of carrier-mediated glutamate export in biotin-limited cells of *C. glutamicum*.

Materials and Methods

Organisms and culture conditions

C. glutamicum ATCC 13032 was grown aerobically on a rotary shaker (150 rpm) at 30°C. With glucose as carbon source (50 g/l) the following mineral salt medium was used: $(NH_4)_2SO_4$ 5 g/l, urea 5 g/l, KH_2PO_4 2 g/l, K_2HPO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 250 mg/l, $FeSO_4 \cdot 7H_2O$ 10 mg/l, $CaCl_2 \cdot 2H_2O$ 10 mg/l, $MnSO_4 \cdot 1H_2O$ 10 mg/l plus trace amounts of $ZnSO_4$, H_3BO_3, $CoCl_2$, $CuSO_4$, NiCl, and $NaMoO_4$, pH 7. Growth on sodium acetate (20 g/l) as sole carbon source required stronger buffering of the medium by either 150 mM potassium phosphate buffer or 180 mM Mops. Glutamate secreting cells (producer cells) were grown in the presence of 0.5–1.0 μg/l biotin. After 14–16 h of incubation, growth arrest and onset of glutamate secretion due to limited biotin supply was reached. Non-producer cells were grown at 200 μg/l biotin. Cell mass was determined by measuring the optical density at 600 nm (OD_{600}), $OD_{600} = 1$ corresponded to 0.34 mg dw/ml for producer cells and 0.36 mg dw/ml for non-producer cells.

Short-term fermentation

Analysis of the secretion process was carried out in short-term fermentations, i.e., with producer cells harvested in early production phase and resuspended in fresh mineral salt medium (for details see Ref. 18). In short term fermentation the pH of the medium quickly rose to 8.0–8.5 due to hydrolysis of urea. If not stated otherwise, glucose was used as carbon source. To ensure optimal oxygen supply, cell suspensions at a cell density of $OD_{600} = 4$–6 were stirred rapidly by a magnetic stirrer at 650 rpm. In this system, producer cells secreted glutamate at a rate of 20–30 nmol/min per mg dw.

Chemicals

[U-^{14}C]Benzoate, [^{14}C]methylamine hydrochloride, inulin[^{14}C]carboxylic acid, [U-^{14}C]glucose, ^3H_2O and ^{86}Rb were purchased from Amersham International (Buckinghamshire, UK). [U-^{14}C]Tetraphenylphosphonium bromide (TPP) was obtained from NEN/DuPont de Nemours (Belgium). Biochemicals were from Boehringer (Mannheim, Germany), all other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

Determination of metabolite concentrations

For the analysis of intra- and extracellular concentrations of metabolites (glutamate, glucose), cells and external medium were separated by silicone oil centrifugation [26]. The supernatant, representing the cell-free external medium, was directly used for metabolite determination. Enzymatic determination of glutamate [27] and glucose [28] was carried out as described elsewhere. Sodium and potassium were measured by atomic absorption spectroscopy.

Preparations of cell extracts for determination of intracellular metabolites

Cells sedimented into perchloric acid by silicone oil centrifugation were resuspended and extracted by sonication. After neutralizing the extract and removing cell debris, denatured protein and precipitated $KClO_4$ by centrifugation, the extract was used for metabolite analysis.

Measurement of the cytoplasmic volume and the components of the protonmotive force

All measurements were carried out as described [29,30]. Separation of the cells from the surrounding medium was achieved by silicone oil centrifugation. For determination of the cytoplasmic volume, inulin[^{14}C]carboxylic acid as membrane impermeable and ^3H_2O as permeable probe were used. For biotin-supplemented, non-producer cells this method consistently yielded values of 2.0 ± 0.2 μl/mg dw. Determination of the cytoplasmic volume of biotin-limited producer cells was more difficult, probably due to different probe binding to the cell surface. In a large number of measurements an average cytoplasmic volume of 1.6 μl/mg dw was found for these cells. This value was generally used for the calculation of intracellular metabolite concentrations. When absolute values for intracellular pools rather than relative changes in the course of an experiment were crucial for interpretation (e.g., experiment of Fig. 1) the cytoplasmic volume was determined repeatedly for the respective cell suspension. In cell sediments of silicone oil centrifugation a ratio of extracellular to intracellular volume of 2:1 was found. Intracellular glutamate was corrected for glutamate originating from the external medium.

The membrane potential was determined from the distribution of [^{14}C]tetraphenylphosphonium cation. Values were corrected for binding of the probe to cell structures using a [^{14}C]TPP-^{86}Rb calibration curve: at different external potassium concentrations (30 μM-300 mM) potassium diffusion potentials in the presence of valinomycin were measured with both probes. ^{86}Rb does not bind to cellular structures. For very low membrane potentials an exact numerical value can not be given due to the large error of the determination in

this range. In these cases the membrane potential will be characterized by indicating a value of ≤ -50 mV.

The pH-gradient was measured by determining the distribution of [^{14}C]benzoic acid ($pH_{in} \geq$; pH_{ex}) or [^{14}C]methylamine ($pH_{in} \leq pH_{ex}$). Correction for probe binding was done by substracting probe bound to cells deenergized by the addition of 20 μM CCCP and 20 μM nigericin.

Internal ATP and ADP concentrations

For the determination of adenine nucleotides, cells were extracted by direct injection of the cell suspension (20 μl) into dimethylsulfoxide (180 μl) [24]. Samples were further diluted (1 : 5) with cold distilled water and kept at −20°C until determination. ATP and ADP were determined using the firefly luciferase assay [31]. For calibration an internal ATP-standard was used.

Glutamate secretion recorded by tracing of radioactive label

At 300 mM external glutamate, glutamate secretion was recorded by tracing radioactive label after addition of [U-^{14}C]glucose. Labelled glucose and labelled glutamate in the external medium were determined in course of the fermentation by the following procedure. Aliquots of cell free medium were subjected to thin-layer chromatography (silica gel 60 plates, Merck, Darmstadt, Germany) using propanol/H_2O (7 : 3, v/v) as solvent [32]. The plates were successively treated with ninhydrin reagent (0.2 g ninhydrin/100 ml butanol, H_2O saturated) and aniline/phthalic acid (100 mM aniline, 100 mM phthalic acid in butanol, H_2O saturated) for staining glutamate and glucose, respectively. Spots were decolorized by H_2O_2, and glucose and glutamate were determined by scintillation counting after removal from the plates.

Determination of potassium efflux

^{86}Rb was used as probe for potassium efflux. Cells were loaded with ^{86}Rb at a low external potassium concentration (0.5 mM KCl). Glutamate secretion during the loading prodedure was drastically reduced by supplying only 0.5 mM of glucose. External ^{86}Rb was equilibrated with the intracellular potassium pool after 40 min of incubation. Glutamate secretion was then started by the addition of glucose. Simultaneously, 30 mM KCl was added to the external medium. Efflux of ^{86}Rb from the cells then represented potassium efflux.

Results

Dependence of secretion on the glutamate concentration gradient

In view of an intracellular glutamate pool of 100–150 mM in producer cells, a substantial gradient is available for glutamate secretion at least at the beginning of fermentation. To test the influence of the chemical gradient as a possible driving force, we measured secretion activity at different initial concentrations of external glutamate (0 mM, 80 mM, 160 mM) (Fig. 1). The applied high external glutamate concentration required the experiment to be carried out at relatively high cell density ($OD_{600} = 9.4$) and for 8 h, to allow secretion of a significant amount of glutamate. Internal

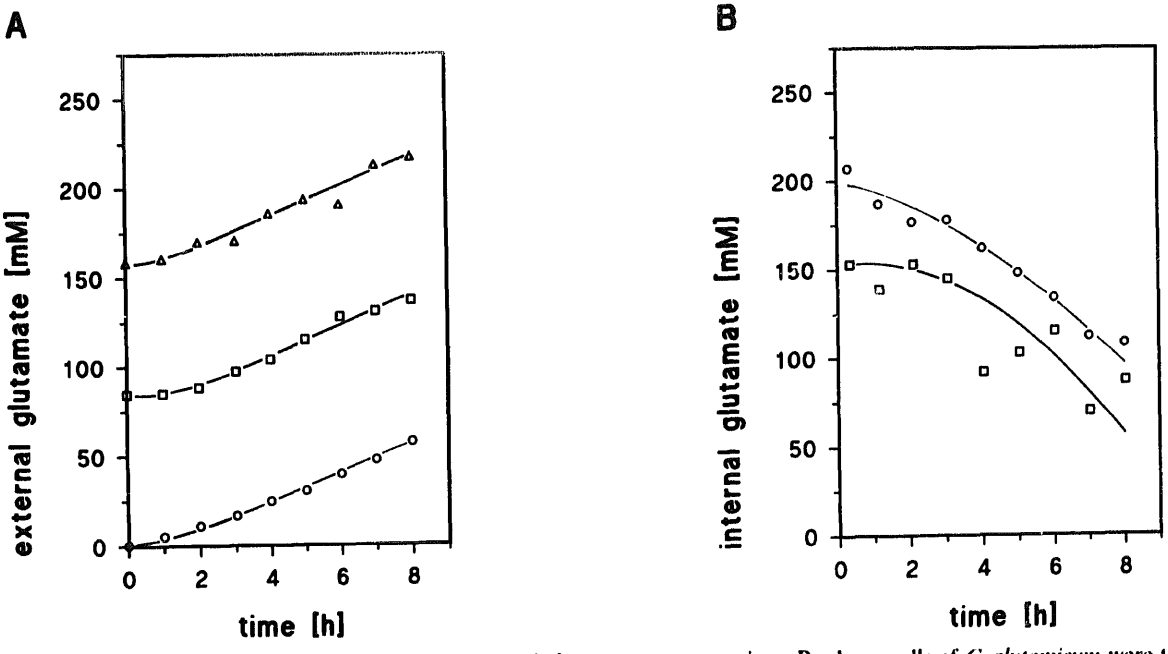

Fig. 1. Glutamate secretion by *C. glutamicum* at different external glutamate concentrations. Producer cells of *C. glutamicum* were transferred to fresh medium (3.2 mg dw/ml) in three separate vessels supplemented with 0 mM (○), 80 mM (□), and 160 mM (△) potassium glutamate. KCl was added to adjust identical ionic strength. Secretion of glutamate into the extracellular medium (A) as well as the intracellular glutamate pool (B) were determined.

glutamate concentrations (Fig. 1B) are shown for cells at 0 mM and 80 mM initial external glutamate concentration. At an initial concentration of 160 mM the experimental error of the determination of internal glutamate was too high to give accurate values. Nevertheless the result of the experiment reported in Fig. 1 is clear: glutamate secretion occurred at identical rate (approx. 40 nmol/min per mg dw), irrespective of the external glutamate concentration. The rate of secretion remained unchanged even when the external glutamate concentration exceeded the internal concentration, a situation at least attained in vessel two (80 mM initial glutamate concentration) after 5 h of fermentation. Glutamate secretion thus is an accumulative process, i.e., it can occur against a concentration gradient.

Influence of the membrane potential on the activity of glutamate secretion

In principle secretion of the glutamate anion could also be driven by the membrane potential (outside positive). Abolishment of the membrane potential by addition of ionophores enabled us to assess its role in the secretion process. As shown in Fig. 2, addition of the protonophore CCCP (50 μM) led to a drastic reduction of the membrane potential from -172 to ≤ -50 mV, without changing the rate of glutamate secretion. An identical result was obtained when valinomycin at high extracellular potassium (300 mM) was used to destroy the electrical potential (data not shown).

In the experiments shown so far (Figs. 1 and 2) either the chemical or the electrical component of the glutamate gradient had been varied. In order to rule

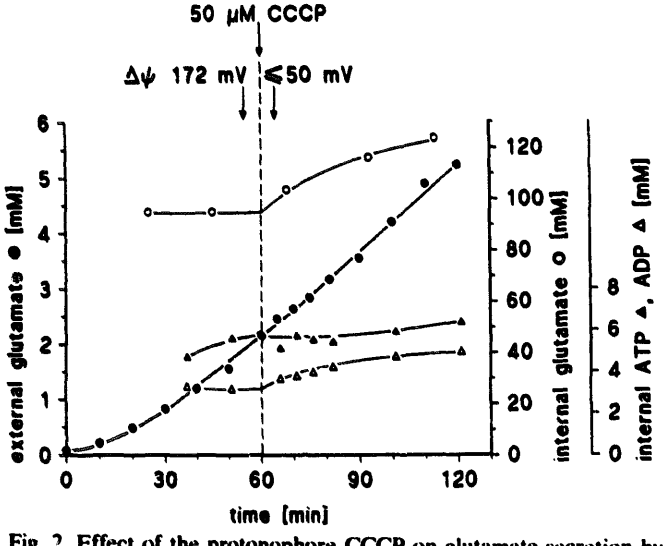

Fig. 2. Effect of the protonophore CCCP on glutamate secretion by *C. glutamicum*. 50 μM CCCP (arrow) was added to producer cells of *C. glutamicum* 60 min after the start of the experiment. The effect on the membrane potential $\Delta\psi$, glutamate secretion (• external glutamate), the cytoplasmic glutamate concentration (o) and the intracellular pools of ATP (▲) and ADP (△) was recorded.

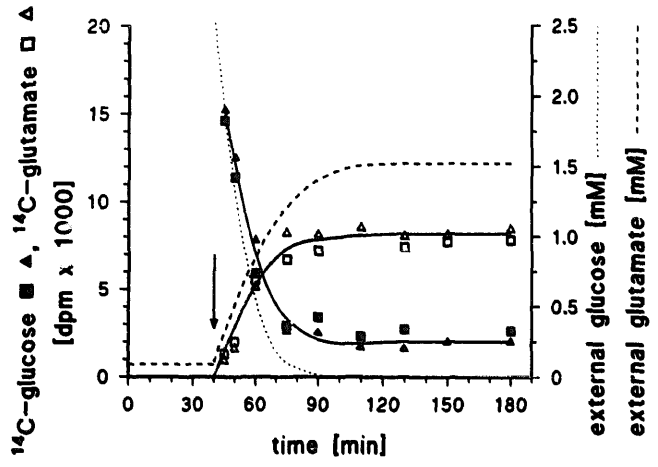

Fig. 3. Influence of simultaneous variation of glutamate concentration gradient and membrane potential on glutamate secretion. 2.5 mM [U-^{14}C]glucose was added (arrow) to producer cells of *C. glutamicum* that had been incubated in two independent culture vessels for 40 min without carbon source. At the same time 300 mM KCl was added to culture 1 (triangles) and 300 mM potassium glutamate plus 50 μM valinomycin to culture 2 (squares). Glutamate secretion was monitored by radioactive and enzymatic determination of both glucose and glutamate in the external medium. Solid symbols: glucose, radioactive; open symbols: glutamate radioactive; dotted line: glucose, enzymatic determination, culture 1 and 2; broken line: glutamate, enzymatic determination, culture 1.

out the possibility that secretion could be driven by either component independently, we used a combination of valinomycin plus 300 mM potassium glutamate to break down both components of the electrochemical glutamate gradient. Glutamate secretion, as determined by tracing the external appearance of glutamate formed from added radioactively labelled glucose (Fig. 3), was identical, irrespective whether the electrochemical glutamate gradient was abolished or not. This demonstrates that glutamate secretion can occur at unchanged rate uphill, against the chemical gradient of glutamate even in the absence of an electrical potential.

Role of monovalent ions (H^+, K^+, Cl^-) in glutamate secretion

The studies described so far assumed glutamate to be transported in anionic form, the predominant glutamate species at the alkaline pH (8–9) in short-term fermentation. Symport of the glutamate anion with a monovalent cation as well as antiport against a monovalent anion would render the transport process electroneutral and thus independent of the membrane potential, as has been observed in the experiments. The most likely candidates for cation symport, protons and potassium ions, and for anion antiport, chloride ions, were investigated.

An involvement of protons in glutamate secretion was tested in the experiment presented in Fig. 4. In medium lacking urea, the external pH decreased con-

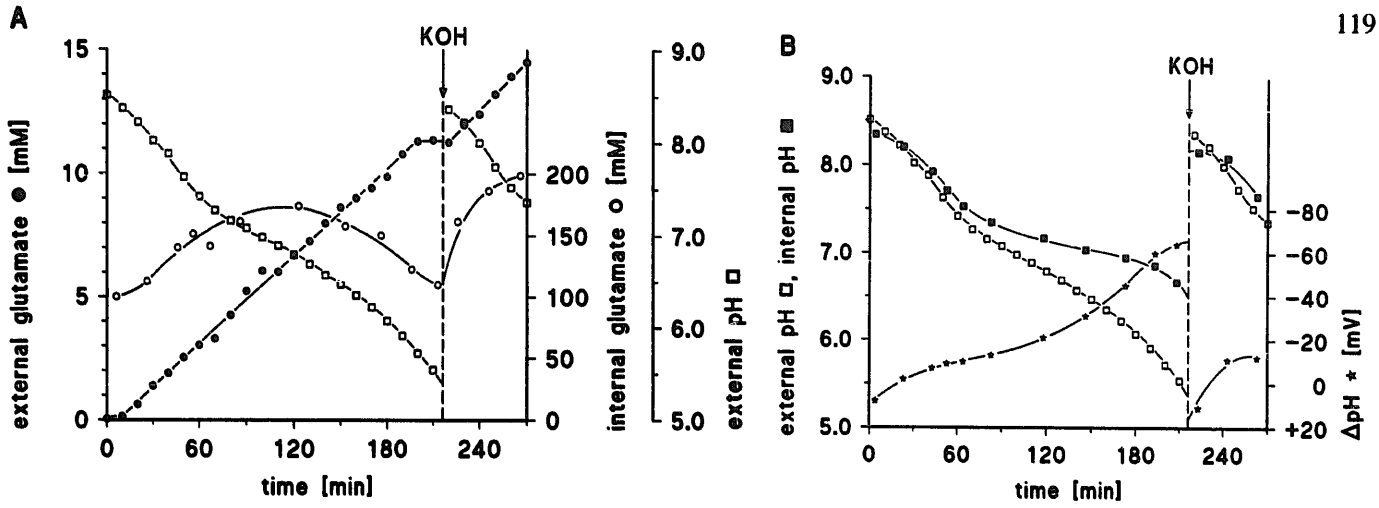

Fig. 4. Dependence of glutamate secretion on variation of the external pH. Fermentation was carried out in mineral salt medium from which urea had been omitted. Ammonium sulfate served as nitrogen source. The continuous drop of medium pH was caused by the metabolic activity of the cells. Internal (○) and external (●) glutamate concentrations (A) as well as the intracellular pH (■) and the ΔpH (*) (B) were determined beside the external pH (□).

tinuously from 8.5 to 5.7 during fermentation due to the metabolic activity of the cells. Thereby the proton gradient changed from +8 mV to −53 mV without affecting glutamate secretion activity. The high intracellular pH (pH_{in} = 8.3) at the beginning of the experiment also had no influence on export velocity. Secretion, however, stopped when the medium was acidified below pH 5.7 causing the intracellular pH to fall below 6.8.

In order to test potassium as a possible symport ion, we directly measured the efflux of potassium ions from cells secreting glutamate (Table I). ^{86}Rb was employed as a probe for potassium fluxes. We confirmed that ^{86}Rb is accepted as substrate by a low-affinity potassium uptake system in *C. glutamicum* (data not shown). In ^{86}Rb-loaded cells, efflux of both glutamate and potassium was determined under different conditions after addition of a carbon source together with an excess of unlabelled potassium to quench the extracellular radioactivity. As the data in Table I show, producer cells lose intracellular ^{86}Rb/potassium far more rapidly than non-producer cells. Potassium efflux, however, is by a factor of 5 slower than the concomittant glutamate efflux in efficiently secreting cells. Reducing the rate of glutamate secretion did not result in an equivalent decrease in potassium efflux.

TABLE I

Potassium efflux from Corynebacterium glutamicum secreting glutamate at different rates

Loading of the cells with ^{86}Rb and recording of ^{86}Rb efflux was carried out as described in Methods and Materials. The reduced rate of glutamate secretion (5 nmol/min per mg dw) was attained by addition of 200 μg/l biotin [25].

Cell type	Biotin concn. (μg/l)	Glutamate secretion rate (nmol/min per mg dw)	Potassium efflux (nmol/min per mg dw)
Producers	1	34	5.1
		23	2.9
		5	3.4
Non-producers	200	0.1	0.5

A possible antiport of glutamate and chloride anions was investigated by measuring glutamate secretion at different extracellular sodium chloride concentrations (data not shown). As in case of protons, only minor changes in secretion activity were observed under these conditions.

Acetate as carbon source for glutamate secretion: the role of ATP

It has been described earlier [33,34] that *C. glutamicum* can also use acetate as carbon source for glutamate secretion. We characterized growth and glutamate secretion of *C. glutamicum* with acetate as carbon source (data not shown). As in glucose grown cells, glutamate secretion was induced only in biotin- starved cells, i.e., when growth had stopped due to lack of biotin. The measured secretion rate was comparable to secretion in glucose grown, biotin-limited cells. The cell density reached with a given limiting biotin concentration was by a factor of two higher in producer cells using acetate as carbon source when compared to producer cells using glucose. This corresponds to observations previously described, that less biotin is needed for optimal glutamate production when acetate instead of glucose is supplied as carbon source [35].

On the basis of the experiments described so far, all possible secondary driving forces were excluded. Since glutamate secretion also functions against a chemical gradient, we had to conclude that chemical energy, e.g., in the form of ATP, must be involved in glutamate secretion. In producer cells metabolizing glucose, the

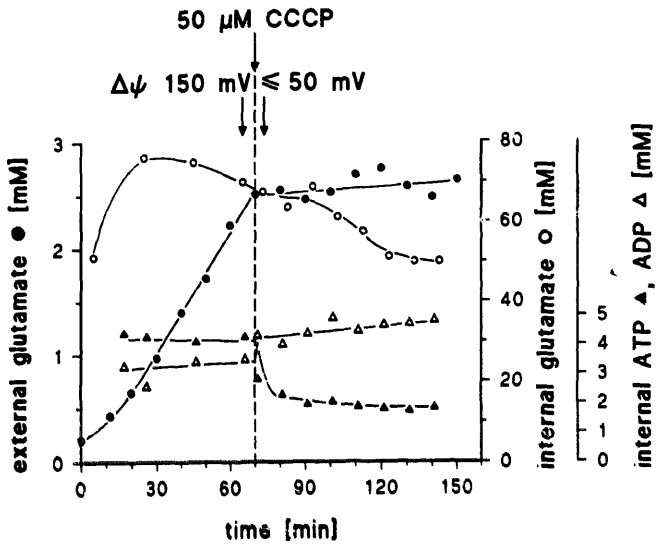

Fig. 5. Influence of the protonophore CCCP on glutamate secretion by *Corynebacterium glutamicum* using acetate as carbon source. After growth on sodium acetate, acetate was supplied as C-source in short-term fermentation. The influence of CCCP addition on membrane potential $\Delta\psi$, the internal (○) and external (●) glutamate concentration and the cytoplasmic pools of ATP (▲) and ADP (△) was monitored.

intracellular ATP pool could be reduced neither by destruction of the membrane potential nor by inhibition of the F_0F_1-ATPase (data not shown). For manipulation of the ATP pool we therefore used cells metabolizing acetate instead of glucose. In these cells ATP is synthesized solely via electron transport phosphorylation, and thus collapsing the membrane potential necessarily must reduce ATP synthesis. The experiment in Fig. 5 demonstrates, that this assumption was correct. Addition of CCCP to acetate grown cells in fact led to

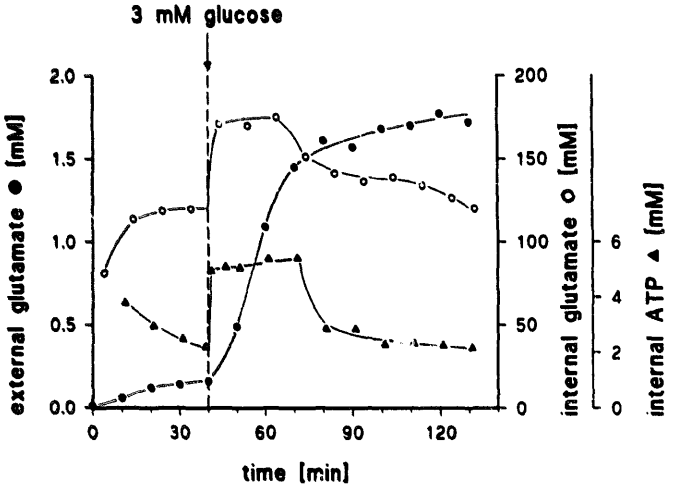

Fig. 6. Dependence of glutamate secretion activity on the supply of C-source. Producer cells of *Corynebacterium glutamicum* were washed twice and were resuspended in mineral salt medium devoid of glucose. After 40 min of incubation, 3 mM glucose was added (arrow). Internal (○) and external (●) glutamate concentrations and the cytoplasmic pool of ATP (▲) were determinded.

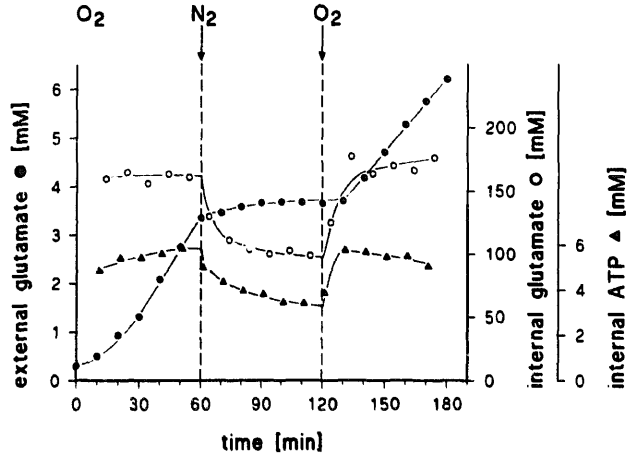

Fig. 7. Dependence of glutamate secretion on the supply of oxygen. Aerobic and anaerobic conditions in the culture were achieved by rapid stirring in contact with air or slow stirring under a N_2 atmosphere, respectively. Internal (○) and external (●) glutamate and the intracellular ATP-pool (▲) were recorded.

a drastic reduction of the cytoplasmic ATP-pool of the cells, thereby inverting the ATP/ADP ratio. At the same time glutamate secretion stopped, whereas the intracellular glutamate concentration decreased only slowly after CCCP addition.

Regulation of secretion activity by the availability of oxygen and carbon source

In a previous paper evidence has been presented that glutamate secretion is effectively regulated in *C. glutamicum* [18]. Secretion is turned off when either the carbon source or oxygen is lacking. We reinvestigated this phenomenon with respect to the role of ATP. As demonstrated in Fig. 6, the onset of secretion following the addition of glucose to starved producer cells was accompanied by a sudden increase of intracellular ATP from 2 mM to more than 4 mM. The ATP pool dropped again to its initial value when glucose was consumed and secretion had stopped. A similar behavior was observed when producer cells were shifted from aerobic to anaerobic conditions (Fig. 7). A high ATP pool was found for efficiently secreting cells under aerobic conditions, while anaerobiosis led to a stop of secretion and concomittantly to a 40–50% decrease of the cytoplasmic ATP concentration. In both experiments a high chemical glutamate gradient existed even when no carbon source or oxygen was available.

Discussion

Evidence for a carrier system involved in glutamate export by biotin-limited cells of *Corynebacterium glutamicum* was already provided by our previously published data [18]. We observed that secretion was strongly regulated, since glutamate efflux could be

stopped in producer cells despite the presence of a high electrochemical gradient of glutamate. Moreover, secretion was specific for glutamate, other amino acids were not excreted. Owing to detailed elucidation of the energetic parameters during glutamate secretion, as reported here, additional strong arguments can now be put forward in favour of a specific glutamate export carrier active in biotin-limited cells. (i) The rate of glutamate secretion was found to be completely unaffected by variation of the extracellular glutamate concentration, secretion proceeded even when the glutamate concentration gradient was inverted (Figs. 1 and 3). (ii) The activity of the secretion system was closely correlated to the energy state of the cells (Figs. 5, 6 and 7). Extracellular acccumulation of glutamate against a concentration gradient can be accomplished neither by diffusion of glutamate through a leaky membrane or a pore nor by carrier mediated facilitated diffusion, mechanisms that could only equilibrate intra- and extracellular glutamate concentrations. The characteristics of glutamate secretion described can be explained solely by a carrier system directly consuming metabolic energy. The source of energy conceivable to drive the export depends on whether the glutamate anion is transported in symport with other ions or on its own. In the former case export of an uncharged complex could use the chemical gradient of a symported ion as driving force, while in the latter case the membrane potential could function as energy source for glutamate export.

Experiments designed to determine the actual transport form of glutamate did not indicate a participation of protons, chloride or potassium ions in the export process and thus point to transport of the glutamate anion. Although elevated efflux of potassium ions was measured from producer cells as compared to non-producer cells (Table 1), this flux can not be mechanistically coupled to glutamate secretion, since it was severalfold slower than glutamate efflux and its rate did not change in parallel to variations of glutamate secretion activity. Independence of secretion from the proton gradient within a wide range (Fig. 4) clearly argues against a symport of the glutamate anion and a proton by a secondary transport system, for which an interdependence of the two parameters has to be expected. The observed stop of glutamate secretion at low extracellular pH can be due to inhibition of the export carrier by simultaneous acidification of the cytoplasm, a phenomenon that has been described for several other transport proteins [36,37]. The fact that an intracellular pH as high as 8.3 (Fig. 4) did not affect the rate of glutamate export is another strong indication that it is in fact the glutamate anion that is transported. The concentration of the undissociated acid at pH 8.3 is reduced to less than 20 μM, which should be well below the K_m of the export system. An affinity for the (internal) substrate in the millimolar rage has been found for the lysine [16] and the isoleucine (unpublished data) export system of *C. glutamicum*. The exact K_m value for glutamate export could not be determined due to the invariably high intracellular glutamate concentration under all conditions tested. Independence of secretion from the membrane potential (Figs. 2 and 3) can only now be interpreted properly: although the membrane potential necessarily exerts a force on the charged substrate, secretion continued unaffected when it was destroyed. Taken together, Figs. 1–3 demonstrate that an energy source apart from the electrochemical glutamate gradient and possibly of significantly higher free energy must be involved in glutamate secretion.

Thus, having secondary forces disqualified, a primary transport mechanism had to be taken into account, for which the most likely energy source would be ATP. Evidence in agreement with a role of ATP was provided by experiments showing a positive correlation of changes in secretion activity and changes in the intracellular ATP-content (Figs. 5, 6 and 7). In glucose-grown cells the intracellular ATP-pool could not be significantly altered by destruction of the membrane potential or by inhibition of the F_0F_1-ATPase, indicating that glycolytic ATP-synthesis prevails under these metabolic conditions. Manipulation of the ATP-pool therefore appeared feasible by $\Delta\psi$-reduction only in cells which are dependent on a non-glycolytic substrate (e.g., acetate) and thus should rely heavily on electron transport phosphorylation for ATP synthesis. Using acetate grown cells it had to be taken into account that the experimental strategy chosen would also reduce carbon flow towards glutamate by reducing $\Delta\psi$-driven acetate uptake [38].

C. glutamicum cells grown on acetate as carbon source showed similar properties with respect to glutamate production as compared to glucose grown cells (data not shown). Stop of secretion following the destruction of the membrane potential in acetate cells (Fig. 5) could be correlated only to the drastic reduction of internal ATP or the ATP/ADP ratio since the intracellular glutamate concentration decreased only slowly. An identical correlation between secretion activity and the intracellular ATP-pool was observed also under conditions leading to metabolic regulation of secretion. The complete and instant stop of secretion when the carbon source or oxygen became limiting also was accompanied by a reduced ATP pool of the cells (Figs. 6 and 7).

A direct role of ATP in the secretion process as energy source for glutamate export can not be proven conclusively in studies using whole cells. It nevertheless represents an obvious possibility after rejection of a secondary mechanism. Correlation between transport activity and the intracellular ATP pool as described

here for glutamate secretion has been observed also for the efflux of a fluorescent probe in *Lactococcus lactis* [39], for which an ATP-driven export system has been proposed. Furthermore, coupling of ATP hydrolysis to export processes could be demonstrated for bacterial heavy metal extrusion [13]. A number of other bacterial exporters most likely employing ATP as source of energy for transport belong to the ABC transporter superfamily [40], e.g., the carriers for α-hemolysin [41] and colicin V [42] in *Escherichia coli*. At present we tend to interprete the correlation between glutamate secretion activity and the intracellular ATP pool in biotin-limited cells of *C. glutamicum* simply as an indication for the strict dependency of secretion on the central energy metabolism of the cells. ATP serves as an indicator for the cellular energy state, it may be either the energy source or a regulator of the export system.

ATP has been demonstrated to be the energy source for glutamate uptake by *C. glutamicum*, which is presumably catalyzed by a binding protein dependent system [43]. A role of the uptake system also in glutamate secretion, as has been proposed in literature [21], can nevertheless be excluded, since the uptake system is repressed in the presence of glucose [36] and thus is present only at a low level in those cells that efficiently secrete glutamate [18].

Recent studies on the mechanism of glutamate secretion in *C. glutamicum* using another condition of inducing secretion, i.e., addition of amine surfactants [24], showed a pattern differing from that described here. For surfactant treated cells the authors could not definitely distinguish whether glutamate production occurred through leakage of glutamate across a permeabilized cell membrane or mediated by a membrane protein. The cells, although not generally leaky, released also aspartate and potassium, the latter at a rate several-fold higher than that of glutamate. The energetic parameters of the cells significantly decreased after surfactant addition. Furthermore, surfactant induced secretion was only a shortlived phenomenon, since secretion ceased after a few minutes due to rapid depletion of the internal glutamate pool. It is therefore not clear whether the detailed analysis given also holds true for the continuous production of glutamate. Besides the obvious difference in the experimental system used, it is thus uncertain, whether direct comparison to our analysis of the secretion process in biotin-limited cells is justified. In this context we would like to emphasize that our studies on mechanism and energy dependence of glutamate secretion in biotin-limited cells have been done under steady-state conditions of production, i.e., with cells that continue to secrete glutamate for substantial periods of time provided that a carbon source is supplied and medium pH is controlled. During production induced by biotin limitation, the energetic integrity of the cells remained intact and secretion was specific for glutamate (see also Ref. 18). The glutamate pool of biotin-limited producer cells, though somewhat lower than in non-producer cells, was constant for prolonged periods of time and the enhanced loss of potassium did not result in depletion of the cellular potassium pool but rather was compensated for by increased potassium uptake. Any perturbation of the energy metabolism inevitably led to stop of secretion in biotin-limited cells.

A full understanding of the physiological meaning of glutamate secretion is still not within reach, but some insight has emerged by comparison with other amino acid export systems in *C. glutamicum* [15–17]. Lysine and isoleucine seem to be excreted whenever these amino acids are present in excess, e.g., when lysine or isoleucine containing peptides are used as carbon source or when these amino acids accumulate intracellularly due to mutations affecting their biosynthetic pathways. Both amino acids, apart from incorporation into protein, can not be converted to other metabolites by either catabolic or anabolic reactions and thus, when present in excess, are useless for the cell. A completely different situation exists in the case of glutamate, as this amino acid has a central role in nitrogen metabolism and as a precursor for other metabolites. Glutamate secretion therefore rather resembles the so-called overflow-metabolism [44]. Glutamate is secreted by the cells under conditions of sufficient carbon and nitrogen supply when concomittantly growth is inhibited due to lack of an essential compound or an impaired integrity of the cell wall/cell membrane complex. The glutamate secretion carrier is activated by exactly those conditions and would thus function in the controlled wasting of energy and carbon.

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References

1 Loubière, P., Goma, G. and Lindley, N.D. (1990) Antonie van Leeuwenhoek 57, 83–89.
2 Michel, T.A. and Macy, J.M. (1990) J. Bacteriol. 172, 1430–1435.
3 Stanfield, S.W., Ielpi, L., O'Brochta, D., Helinski, D.R. and Ditta, G.S. (1988) J. Bacteriol. 170, 3523–3530.
4 Styrvold, O.B. and Strom, A.R. (1991) J. Bacteriol. 173, 1187–1192.
5 Nies, D.H. and Silver, S. (1989) J. Bacteriol. 171, 896–900.
6 Silver, S., Nucifora, G., Chu, L. and Misra, T.K. (1989) TIBS 14, 76–80.
7 Yamaguchi, A., Adachi, K., Akasake, T., Ono, N. and Sawai, T. (1991) J. Biol. Chem. 266, 6045–6051.

8 Midgley, M. (1987) Microbiol. Sci. 4, 125–127.
9 Konings, W.N., Poolman, B. and Driessen, A.J.M. (1992) FEMS Microbiol. Rev. 88, 93–108.
10 Ten Brink, B. and Konings, W.N. (1980) Eur. J. Biochem. 111, 59–66.
11 Otto, R., Sonnenberg, A.S.M., Veldkamp, H. and Konings, W.N. (1980) Proc. Natl. Acad. Sci. USA 77, 5502–5506.
12 Neyfakh, A.A., Bidnenko, V.E. and Chen, L.B. (1991) Proc. Natl. Acad. Sci. USA 88, 4781–4785.
13 Kaur, P. and Rosen, B.P. (1992) Plasmid 27, 29–40.
14 Kinoshita, S. (1985) in Biology of Industrial Microorganisms (Demain, A. and Solomon, N., eds.), pp. 115–142, Benjamin/Cummings, Menlo Park.
15 Ebbighausen, H., Weil, B. and Krämer, R. (1989) Appl. Microbiol. Biotechnol. 31, 184–190.
16 Bröer, S. and Krämer, R. (1991) Eur. J. Biochem. 202, 131–135.
17 Bröer, S. and Krämer, R. (1991) Eur. J. Biochem. 202, 137–143.
18 Hoischen, C. and Krämer, R. (1989) Arch. Microbiol. 151, 342–347.
19 Demain, A.L. and Birnbaum, J. (1968) Curr. Top. Microbiol. Immunol. 46, 1–25.
20 Milner, J.L., Vink, B. and Wood, J.M. (1987) CRC Crit. Rev. Biotechnol. 5, 1–47.
21 Clement, Y., Escoffier, B., Trombe, M.C. and Lanéelle, G. (1984) J. Gen. Microbiol. 130, 2589–2594.
22 Shio, I., Otsuka, S. and Takahashi, M. (1961) J. Biochem. 51, 56–62.
23 Huchenq, A., Marquet, M., Welby, W., Montrozier, H., Goma, G. and Lanéelle, G. (1984) Ann. Microbiol. (Inst. Pasteur) 135B, 53–67.
24 Duperray, F., Jezequel, D., Ghazi, A., Letellier, L. and Shechter, E. (1992) Biochim. Biophys. Acta 1103, 250–258.
25 Hoischen, C. and Krämer, R. (1990) J. Bacteriol. 172, 3409–3416.
26 Klingenberg, M. and Pfaff, E. (1967) Methods Enzymol. 10, 680–684.
27 Beutler, H.O. and Michal, G. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H.U., ed.), Vol. 2, pp. 1753–1759, Verlag Chemie, Weinheim.
28 Kunst, A., Draeger, B. and Ziegenhorn, J. (1983). in Methods of enzymatic analysis (Bergmeyer, H.U., ed.), Vol. 6, pp. 163–172, Verlag Chemie, Weinheim.
29 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569.
30 Kashket, E.R. (1985) Annu. Rev. Microbiol. 39, 219–242.
31 Schimz, K.L, Rütter, B. and Tretter, M. (1981) Adv. Biotechnol. 1, 457–462.
32 Stahl, E. (1969) Thin-Layer Chromatography, 2nd Edn., Springer, Berlin, Heidelberg, New York.
33 Kanzaki, T., Kitano, K., Sumino, Y. and Okazaki, H. (1972) J. Agr. Chem. Soc. Jap. 46, 95–101.
34 Kitano, K., Sugiyama, Y. and Kazaki, T. (1972) J. Ferment. Technol. 50, 182–191.
35 Kinoshita, S. and Tanaka, K. (1972) in The microbial Production of Amino Acids (Yamada, K., Kinoshita, S., Tsunoda, T. and Aida, K., eds.), pp. 263–324, Kodansha, Tokyo, John Wiley and Sons, New York.
36 Krämer, R., Lambert, C., Hoischen, C. and Ebbighausen, H. (1990) Eur. J. Biochem. 194, 929–935.
37 Poolman, B., Hellingwerf, K.J. and Konings, W.N. (1987) J. Bacteriol. 169, 2272–2276.
38 Ebbighausen, H., Weil, B. and Krämer, R. (1991) Arch. Microbiol. 155, 505–510.
39 Molenaar, D., Bolhuis, H., Abee, T., Poolman, B. and Konings, W.N. (1992) J. Bacteriol. 174, 3118–3124.
40 Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. (1992) Nature 346, 362–365.
41 Blight, M.A. and Holland, I.B. (1990) Mol. Microbiol. 4, 873–880.
42 Gilson, L., Mahanty, H.K. and Kolter, R. (1990) EMBO J. 7, 3875–3884.
43 Krämer, R. and Lambert, C. (1990) Eur. J. Biochem. 194, 937–944.
44 Neijssel, O.M. and Tempest, D.W. (1979) Microbial Technology, Symp. Soc. Gen. Microbiol. 29, 53–82.