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Mechanism and energetics of amino-acid transport in coryneform bacteria

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1. Introduction

Uptake of substrates is an essential function for every living cell. The significance of membrane transport, however, is not simply to supply the cell with nutrients and other substrates but also to remove metabolic waste products. Thus, the secretion of metabolites is also a common function in bacteria. There are a variety of secretion processes of which the physiological significance is evident. The most obvious are excretion of fermentation end products, i.e., monocarboxylic and dicarboxylic acids, alcohols and ketones. These products either cross the plasma membrane passively (e.g., some alcohols, acetone), or they are excreted by secondary mechanisms (e.g., lactate and succinate) [1]. Another common kind of product secretion is precursor/product antiport mechanisms [2]. A different class of excreted products are toxic substances which are actively removed from the cell. This can be catalyzed by ATP-dependent primary systems, e.g., extrusion of heavy metal ions, or by secondary systems, e.g., extrusion of tetracycline. Further processes by which a variety of solutes is excreted function in response to hypoosmotic stress. In the last few years, we have focused on the elucidation of mechanisms by which *Corynebacterium glutamicum* is able to secrete amino acids into the medium, e.g., lysine [3], isoleucine [4] or glutamate [5].

In contrast to uptake, metabolite secretion has not been studied extensively, except in the above mentioned examples, although it is a common phenomenon in many bacteria. In spite of an early conceptual approach by interpreting metabolite efflux as a carrier-mediated process in the case of lactate secretion by *Lactococcus lactis* [6], this concept was in general not accepted for metabolite efflux. The main reason was the fact that frequently secretion of these solutes occurred under 'unphysiological' conditions (deregulated mutants, elimination of control points, increased internal concentrations). It was difficult to assume that the cell should provide carrier mechanisms for this particular situation. There is, however, no doubt about the economic significance, since the majority of amino acids (currently $\geq 600\,000$ tons/year) are produced by using microorganisms such as *Escherichia coli*, *Serratia marcescens* and especially coryneform bacteria. In the following, several examples of transmembrane amino-acid flux will be discussed with respect to functional, regulatory and energetic aspects of the interaction of secretion and uptake, i.e., that of glutamate, isoleucine, threonine, lysine, and aromatic amino acids in *C. glutamicum*. The selection of these solutes covers a wide spectrum of biochemical properties with respect to both charge and polarity.

2. Mechanism and energetics of amino-acid uptake and efflux

Numerous amino-acid uptake systems with various mechanisms have been characterized in bacteria. For amino-acid secretion, three mechanisms have been suggested [7]. (a) Amino acids accumulated in the cytosol under particular metabolic conditions may pass

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the membrane by diffusion or by (osmotically controlled?) pores (leak hypothesis). (b) Efflux of amino acids may be catalyzed via functional inversion of uptake systems which are available for the respective amino acid (inversion hypothesis). (c) Specific secretion carriers for amino-acid efflux in *C. glutamicum* may be present (carrier hypothesis) [5].

The kinetics and the energetics of amino-acid transport depend on the fact whether (i) an uptake carrier and/or (ii) a secretion system is present. (iii) It furthermore depends on the hydrophobicity of the particular amino acid which determines whether diffusion-controlled fluxes may occur. (iv) The charge of the solute determines whether the membrane potential affects its transmembrane movement. (v) Furthermore, a variety of driving forces may be important, due to cotransported ions (secondary transport) or chemical energy (primary transport). Table 1 gives an overview of the topics discussed in this contribution with respect to bacterial amino-acid transport.

3. Glutamate uptake and secretion in *C. glutamicum*

Glutamate uptake in *C. glutamicum* was characterized as a primary, ATP-dependent mechanism [8]. Thus, this system is another example of the recently discovered binding protein-dependent transport systems in Gram-positive bacteria. The activity of the glutamate uptake carrier is modulated by internal pH and K^+ concentration. Its expression is tightly regulated and subject to both repression by glucose and induction by glutamate. Recently, we identified, cloned and sequenced the genes of the glutamate uptake system in *C. glutamicum* (Fig. 1). As a typical binding protein-dependent transport system, it includes a binding protein, two hydrophobic membrane-inserted subunits and an energy-coupling component with two ATP binding sites. The binding protein contains an N-terminal cysteine within a typical motif of six amino acids, which is indicative for membrane-insertion by a lipid anchor. This was corroborated by the finding that globomycine,

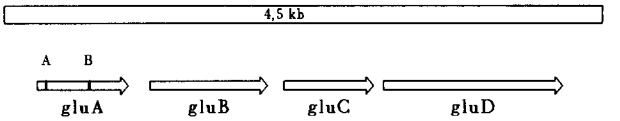

Fig. 1. Gene map of the *glu* operon in *C. glutamicum*. The four genes mean *gluA*, ATP-binding protein; *gluB*, binding protein; *gluC* and *gluD*, membrane protein subunits. The two boxes (A and B) in *gluA* represent the ATP-binding sites.

an inhibitor of the lipoprotein-specific leader peptidase, inhibited the function of the glutamate uptake system. Interestingly, the V_{max} was not severely affected; however, the apparent K_m increased by more than three orders of magnitude. Thus, although transport could barely be measured by conventional uptake tests, the bacteria still grew in media with high glutamate content.

The metabolic conditions leading to glutamate secretion are clearly different from those responsible for the secretion of other amino acids (see below). This is due mainly to the fact that the cytosolic glutamate concentration in *C. glutamicum* (like many other bacteria) is very high. Levels of up to 500 mM are found, whereas those of other amino acids are in general in the low mM range. Thus, whereas in general the cytosolic pool has to be increased in order to trigger amino-acid secretion, the internal glutamate pool is always high. Consequently, other factors must regulate glutamate secretion. We found that the conditions which lead to glutamate secretion resemble those of a so-called 'overflow metabolism' [9]. This type of massive metabolite excretion is observed whenever (i) the carbon and energy source is present in excess, (ii) growth of the microorganisms is limited by lack of some essential nutrient or other component, and (iii) the uptake of substrate cannot be effectively regulated. However, since a mechanistic correlation of the physiological characteristics of overflow metabolism with the observed secretion events has not been defined so far, it does not provide a helpful explanation for understanding the factor(s) triggering glutamate secretion.

Table 1
Different solute fluxes in amino-acid transport in *C. glutamicum*

Amino acid	Diffusion rate constant (min^{-1})	Mechanism and V_{max} ($\mu mol\ min^{-1}\ (mg\ dw)^{-1}$)	
		uptake system	secretion system
Glutamate	< 0.001	primary (1,5–16) [a]	(primary?) (25)
Lysine	< 0.004	antiport (0.2)	OH^- symport (3–11) [a,b]
Isoleucine	0.08	Na^+ symport (0–2) [a]	secondary (2–20) [a]
Threonine	0.006	Na^+ symport (2)	secondary (0–5) [a]
Phenylalanine	0.45	secondary (?)	?

[a] Dependent on the state of induction/repression (see text).
[b] Dependent on properties of different strains (see text).

Glutamate secretion differs from the mechanisms described for other amino acids in another fundamental aspect. Glutamate secretion has to be induced by procedures which change the physical state of the membrane by either limiting phospholipid synthesis or directly influencing the membrane [10,11]. Detailed investigations proved that (i) during glutamate secretion the electrochemical potential stays high and the passive permeability of the membrane is not significantly changed [12], (ii) glutamate can be secreted against an existing chemical gradient [5], (iii) the secretion carrier is not driven by the membrane potential, the pH-gradient or other ion gradients [5], (iv) secretion is independent of the glutamate uptake system. A correlation of glutamate secretion with the phosphorylation potential of the cell has been found [5], although a primary mechanism could not be proven. As a possible explanation for some of these observations the modulation of a putative carrier by the state of the membrane (molecular order, viscosity) has been put forward. We studied this question by introducing a controlled change in the membrane state, e.g., addition of local anaesthetics. Addition of tetracaine led to secretion with similar activity as that observed under typical 'production' conditions (Krämer, R., unpublished data). In another procedure, effective secretion could be obtained by changing the membrane order in response to the presence or absence of divalent cations. In all these experiments, the energetic state of the cells and the permeability properties of the membrane were unchanged.

We furthermore observed that the activity of glutamate secretion was dependent on the difference in osmotic pressure across the plasma membrane (Table 2). Secretion was stimulated by increasing amounts of the fluidizing agent tetracaine, as well as by hypoosmotic conditions. Bacteria in general react to hypoosmotic stress by releasing various solutes, e.g., K^+, proline, glutamate, as well as typical compatible solutes like glycine betaine or trehalose. The extremely rapid release of these substances as observed in *E. coli* after an osmotic downshock is currently interpreted as being mediated by stretch-activated channels [13], although the nature of these channels has not been revealed so far. The slow efflux of glycine betaine in *Salmonella typhimurium* and in *E. coli* [14], on the other hand, is mediated by specific secretion systems. Also the uptake systems for proline and betaine in *E. coli* are presumably regulated by the cell turgor [15]. The functional significance of the glutamate secretion system thus seems to be correlated to osmoregulation, similar to the above mentioned examples.

Table 2
Dependence of glutamate secretion on tetracaine addition and on the difference of internal and external osmolarity

Tetracaine concentration (mM)	Medium osmolarity after shift (mosM)	Glutamate secretion rate (μmol min^{-1} (mg dw)$^{-1}$)	Internal glutamate (mM)
0.5	170	1.5	190
0.7	170	4.5	175
1.1	170	20.5	140
0.7	85	21	140
0.7	170	4.5	175
0.7	340	< 0.5	210
1.1*	85	10 *	40 *
1.1	170	20.5	140
1.1	340	< 0.5	205

Upon addition of tetracaine, the cells were shifted from a medium with 170 mosM to media with the indicated osmolarity. Both the rate of active glutamate secretion and the cytosolic glutamate concentration were determined after the osmotic shift. The integrity of the membrane was not significantly changed, except in the experiment marked with an asterisk.

Table 3
Counteracting glutamate uptake and secretion in *C. glutamicum* cells grown on glutamate

Time (min)	Rates (μmol min^{-1} (mg dw)$^{-1}$)			
	uptake	secretion	efflux	futile cycling
10	16	0	0	0
30	12	8	0	8
60	9	15	6	9
90	6	23	17	6
120	5	25	20	5
150	11	< 3	0	< 3

At 15 min, membrane-acting agent was added; at 135 min, it was removed again. Efflux = secretion-uptake (only positive values).

It is now interesting for energetic aspects, whether the presence of glutamate uptake and secretion leads to futile cycling of the solute and thus to a waste of energy. Under normal conditions, this is obviously avoided. First, passive diffusion of glutamate is very low and such fluxes can be neglected. Second, during growth on glucose, the glutamate uptake system is repressed. In 'production' conditions, the low glutamate uptake activity does therefore not lead to significant futile cycling. Third, it is obvious that the secretion system is tightly regulated. Under normal growth conditions, the primary active glutamate uptake system leads to extraordinarily high accumulation ratios up to $2 \cdot 10^5$ [8]. The presence of glutamate secretion under these conditions would prevent such high solute gradients. On the other hand, conditions can be defined, where futile cycling is observed (Table 3). When *C. glutamicum* cells, grown on glutamate, i.e., with fully induced glutamate uptake system, are treated with membrane-acting agents as described above, a shift from an 'uptake situation' to a 'secretion situation' occurs. During this functional shift, effective futile cycling is observed.

4. Mechanism and energetics of isoleucine uptake and secretion

It is in general difficult to quantitate carrier mediated transport if the transported substrate, like isoleucine, is amphiphilic thus being able to cross the membrane also passively. In this case all possible mechanisms have to be considered, namely carrier-mediated isoleucine secretion and uptake as well as diffusion-controlled fluxes. It was necessary to develop appropriate methods for studying isoleucine flux across the membrane of *C. glutamicum*. For this purpose, we used the addition of defined peptide mixtures under conditions of induced peptide uptake. In contrast to isoleucine synthesis, isoleucine formation by hydrolysis of dipeptides is not regulated. This led to increased internal steady-state levels of isoleucine, the actual value of which is determined by both the influx into and efflux from the isoleucine pool. The level of the cytosolic isoleucine pool could thus be modulated by using various peptide mixtures with different content of isoleucine.

Carrier-mediated isoleucine uptake in *C. glutamicum* is catalyzed by a secondary, Na^+-coupled mechanism [16], similar to branched-chain amino-acid uptake systems in other bacteria. An unusual property of this system is the regulation of its synthesis by internal isoleucine. Only in the presence of about 0.5 mM cytosolic isoleucine or higher, the uptake system becomes expressed [17]. In isoleucine auxotrophic strains, therefore, an uptake system cannot be detected. Furthermore, evidence for the presence of an energy-dependent isoleucine secretion carrier, different from the uptake carrier was provided [4]. The secretion carrier shows the basic properties of carrier-mediated processes, i.e., saturation, specificity and inhibition by covalent reagents. The mechanism of isoleucine secretion resembles that of the lysine carrier in *C. glutamicum* [7]. Lysine efflux has been described in detail as a secondary symport with two hydroxyl ions, thus leading to an outside accumulation of lysine driven by the membrane potential [3]. Interestingly, similar to lysine [18], in wild type cells a certain threshold concentration of internal isoleucine was observed below which no secretion occurred (Fig. 2). The different isoleucine fluxes (diffusion, carrier-mediated secretion and uptake) have been determined independently (Fig. 2). Under the conditions in which secretion, uptake and diffusion take place simultaneously, a futile cycle is created.

Different situations with respect to transmembrane isoleucine flux can be envisaged in dependence of various internal isoleucine concentrations (Fig. 2). At very low concentrations, the secretion system is inactive and the state of induction of the uptake system is important. When the internal concentration exceeds

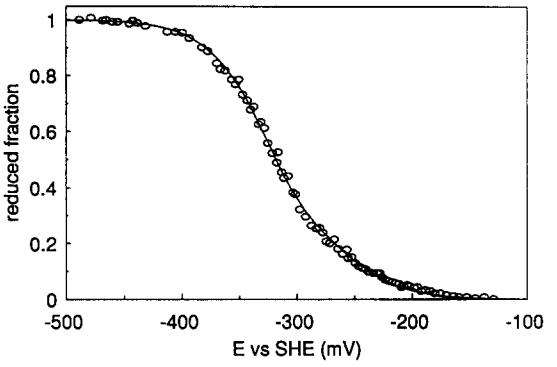

Fig. 2. Isoleucine fluxes in *C. glutamicum* under conditions of partially induced isoleucine secretion. Dependent on the growth conditions, the activity of the uptake system varies between 1 and 2 μmol min^{-1}(mg dw)$^{-1}$, that of the secretion system between 3 and 25 μmol min^{-1}(mg dw)$^{-1}$. A positive sign indicates the efflux direction, a negative sign stands for uptake; then: efflux = secretion + uptake + diffusion (external isoleucine concentration $\ll$ internal isoleucine).

0.5 mM, the isoleucine uptake system becomes expressed [18] because it is advantageous for the cell to recover the small amounts of isoleucine which are lost by diffusion. This idea has been designated 'cyclic retention hypothesis'. When, because of particular conditions of nutrient availability, the cytosolic isoleucine concentration is increased above a certain level (about 8 mM for activation), the cell obviously realizes that this concentration is not any more advantageous. Consequently, enhanced synthesis of the secretion carrier is measured, as well as stimulation of its activity. Even under these conditions, however, if the energy and carbon supply of the cell becomes limiting, *C. glutamicum* switches off the secretion carrier, in spite of high internal isoleucine. It is of course interesting to know in which metabolic situation this complicated regulation pattern is of physiological importance. We found that the significance of isoleucine secretion is based on the same principles as described for lysine. Growth on peptides composed of certain amino acids, which cannot be metabolized by *C. glutamicum*, leads to massive excretion of these amino acids [18].

5. Uptake and secretion of threonine and phenylalanine in *C. glutamicum*

Threonine belongs to the same class of amino acids as lysine and isoleucine with respect to secretion properties. *C. glutamicum* possesses an Na^+-dependent secondary threonine uptake system (Table 1), as well as a secondary threonine secretion system, the activity of which is effectively regulated. In the case of threonine the activity of the secretion system is not much higher than that of the uptake system. Thus, although high internal threonine levels can be obtained in appropri-

ate mutant strains, net efflux of this amino acid never reaches values as observed for the other amino acids mentioned here. In this case, *C. glutamicum* uses another way of getting rid of high internal amino-acid (threonine) concentrations, namely degradation to glycine.

More hydrophobic amino acids may cross the membrane by diffusion, e.g., phenylalanine. The measured first order rate constant of diffusion (in Table 1) leads to efflux rates matching those of lysine secretion even at moderate internal phenylalanine concentration (10 mM). In spite of this high rate of passive permeation, we found that *C. glutamicum* possesses uptake systems also for these hydrophobic solutes, as do other bacteria, such as *E. coli*.

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