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Biochimica et Biophysica Acta 1196 (1994) 14–20

BBA Biochimica et Biophysica Acta

Mechanism of alanine excretion in recombinant strains of *Zymomonas mobilis*

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Received 23 June 1994

Abstract

A thiamine-auxotrophic strain of *Zymomonas mobilis* (CP4thi/pZY73), in which the *alaD* gene of *Bacillus sphaericus* coding for the alanine dehydrogenase was expressed, synthesizes and excretes alanine at high rates after thiamine starvation and in the presence of high external ammonium concentrations. The mechanism of alanine excretion was studied in this recombinant *Zymomonas mobilis* strain. Under production conditions the internal alanine concentration reached values of up to 280 mM and excretion rates of up to 140 nmol min^{-1} mg dry $mass^{-1}$ were obtained. The membrane integrity and the energetic properties of the cells remained intact and were comparable to growing wild-type cells. Unspecific leakage of solutes was not observed. We did not find any indication of a carrier-mediated excretion of alanine, since typical properties of this type of mechanism, i.e., saturation at increasing internal substrate concentration, substrate specificity and functional inhibition were absent. Furthermore, a counterflow maximum, which would indicate the involvement of a carrier protein, was not observed either. Consequently, alanine excretion in recombinant *Z. mobilis* cells is interpreted as mediated by simple diffusion through the intact cytoplasmic membrane at high rates (diffusion constant 10^{-8} l s^{-1} mg dry $mass^{-1}$ or 0.28 min^{-1}). For comparison, the diffusion constant for alanine efflux was also measured in *Corynebacterium glutamicum* cells and the values obtained were significantly lower than those determined in *Z. mobilis*. The consequences of this finding are discussed.

Keywords: Amino acid excretion; Alanine; Recombinant strain; (*Z. mobilis*)

1. Introduction

Zymomonas mobilis is unique among bacteria in fermenting sugar anaerobically by the Entner–Doudoroff pathway and catabolizing pyruvate to ethanol by pyruvate decarboxylase [1–3]. The biotechnological use of this organism is limited by its narrow substrate and product spectrum (for review see [4]). In an attempt to broaden the product spectrum of *Z. mobilis*, the cloning and expression of an alanine dehydrogenase from *Bacillus sphaericus* in a thiamine-auxotrophic strain of *Z. mobilis* was recently reported [5]. The alanine dehydrogenase catalyzes the reversible deamination of L-alanine to pyruvate and ammonia using NAD^+ as a cofactor. Since the pyruvate decarboxylase would draw off the major fraction of the available pyruvate, a limitation of the thiamine supply is necessary to redirect a significant portion of the carbon flux to alanine. Under these conditions, the activity of the (thiamine-dependent) pyruvate decarboxylase is decreased and this strain then effectively synthesizes and excretes alanine [5].

In the recombinant strain *Z. mobilis* CP4thi/pZY73, alanine excretion rates were observed which by far exceeded amino acid excretion activity as observed in amino-acid-producing bacteria such as *Corynebacterium glutamicum* [6–9]. It was thus interesting to elucidate the mechanism of alanine excretion in *Z. mobilis* under alanine production conditions. In *Z. mobilis* we found that alanine excretion is mediated by simple diffusion. Specific excretion systems for several amino acids have recently been characterized in the amino acid producer *C. glutamicum* [9]. Passive diffusion of amino acids in bacteria has been studied especially for proline [10,11], but also for several other amino acids, including alanine ([12,13], for review see [14]). With the exception of proline, however, the excretion reaction has not been quantitatively investigated.

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SSDI 0005-2736(94)00201-0

2. Materials and methods

2.1. Organisms and growth conditions

The construction of the thiamine-auxotrophic strain *Zymomonas mobilis* CP4thi/pZY73 carrying the *alaD* gene from *B.sphaericus* IFO3525 is described elsewhere [5,15]. *Z.mobilis* strains were grown anaerobically overnight in complex medium at 30°C as described earlier [5,16,17]. The medium contained (per liter) 50 g of glucose, 10 g of Bacto yeast extract, 1 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 0.5 g of $MgSO_4 \cdot 7\ H_2O$ (pH 5.5.). Cells were harvested at a cell density of 0.35 to 0.5 mg (dry mass) per ml, washed twice in sterile mineral salts medium (MM), resuspended in MM at a cell density of 0.1 mg (dry mass) per ml, and incubated for 16 h at 30°C. Mineral medium (MM) was modified [5] and contained 1.0 g of $MgSO_4 \cdot 7\ H_2O$, 3.48 g of KH_2PO_4, 0.2 g of citric acid monohydrate, 0.01 g of $FeSO_4 \cdot 7\ H_2O$, 19.52 g of 2-(*N*-morpholino)ethanesulfonic acid and 1.98 g of $(NH_4)_2SO_4$ (pH 6.0). After sterilization, calcium pantothenate and biotin were added to a final concentration of 1.5 mg of each per liter. Finally cells were harvested and suspended in the 'production medium', i.e., mineral medium (MM) supplemented with a final concentration of 100 mM ammonium acetate, pH 7.0, usually at about 2.5–3.5 mg (dry mass) per ml. If not otherwise specified, 5% glucose (w/w) was added as carbon source. *Corynebacterium glutamicum* cells were grown as described previously. Internal amino acid concentrations were increased using the peptide feeding method [18].

2.2. Chemicals

Radiochemicals were purchased from Amersham International (Amersham, Buckinghamshire, UK). The following labeled compounds were used: [U-^{14}C]alanine, [U-^{14}C]glutamic acid, [U-^{14}C]taurine, ^3H_2O, [^{14}C]thiocyanate (potassium salt). Biochemicals were from Boehringer (Mannheim); all other chemicals were of analytical grade and were obtained from Merck (Darmstadt) or Sigma (St. Louis, MO).

2.3. Determination of cytoplasmic volume and membrane potential

The cytoplasmic volume was measured by determining the distribution of [^{14}C]taurine and ^3H_2O [19]. Cells were separated from the surrounding medium by silicone oil centrifugation [20]. Details of this method are described elsewhere [21]. The membrane potential was quantified by determining the distribution of KS^{14}CN, as described in [17]. The cytosolic concentration of SCN^- decreases with increasing value of the membrane potential (negative inside). Cells were deenergized by the addition of 100 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or by glucose starvation [17].

2.4. Determination of internal and external alanine by HPLC

200 μl of the cell suspension was separated by silicone oil centrifugation and extracted in perchloric acid. The supernatant was used to determine the external alanine concentration. The extracts were sonicated, neutralized by adding 25 μl of a solution containing 5 M KOH/1 M triethanolamine, kept on ice for 1 h and centrifuged. The resulting supernatants were used to determine the internal amino acid concentration. Alanine was measured fluorometrically after reverse-phase high-performance liquid chromatography (HPLC) with precolumn derivatization using *ortho*-phthalaldehyde in an HP 1090 chromatograph. Details are described elsewhere [21].

2.5. Enzymatic determinations

Cells from the exponential growth phase were harvested by centrifugation and resuspended in MM. An aliquot of 0.4 ml was sonicated on ice. Cell debris was sedimented at $100\,000 \times g$ for 30 min. The resulting supernatant was used for alanine determination by oxidative deamination to pyruvate with alanine dehydrogenase [22]. The ATP content was detected by the firefly luciferin-luciferase assay [23]. Internal and external standards were used to calculate the ATP concentration. Details are given elsewhere [24]. Protein was determined according to [25] using bovine serum albumin as standard.

2.6. Calculation of rates and rate constants

The alanine excretion rates (in nmol ml^{-1} min^{-1}) or the specific excretion rates (in nmol min^{-1} mg dry $matter^{-1}$) were measured directly. By assuming a first-order process (diffusion) these rates can be converted into first-order rate constants (in min^{-1}) or diffusion rate constants (in $\mu l\ min^{-1}$ mg dry $matter^{-1}$) by taking into account the measured internal volume of the cells (in general 2–2.5 μl per mg dry matter) under the respective experimental conditions in combination with the measured internal alanine concentration.

3. Results

When provided with glucose and high external ammonium concentrations, in the absence of thiamine the recombinant strain *Zymomonas mobilis* CP4thi/pZY73 synthesized alanine at high rates leading to significant internal alanine concentrations and to remarkably high excretion rates [5] (Fig. 1). Within a few minutes, the cytosolic alanine concentration rose to values higher than 200 mM.

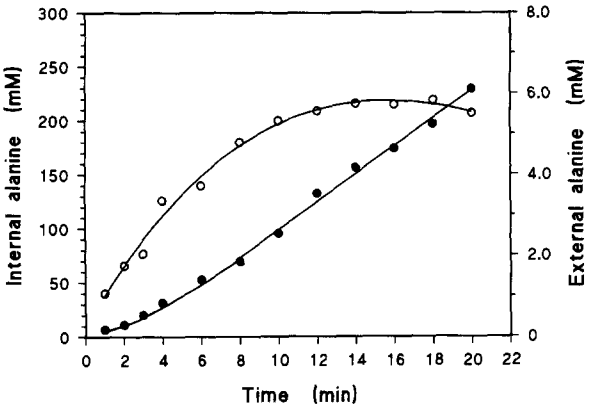

Fig. 1. Internal (○) and external (●) alanine concentration after the addition of thiamine-starved *Z. mobilis* CP4thi/pZY73 cells to production media (5% glucose, 100 mM ammonium acetate) at 37°C. Cell concentration was 3 mg dm ml^{-1}.

Correspondingly, excretion rates exceeding 100 nmol min^{-1} mg dry $matter^{-1}$ could be observed. After about 10 min a steady state was reached leading to constant high excretion rates. The variation in the time scale of the rise in internal alanine (Figs. 1 and 2) is due to somewhat different efficiencies of the thiamine starvation procedure, which leads to a variation of the share of carbon flow into the direction of alanine and ethanol, respectively.

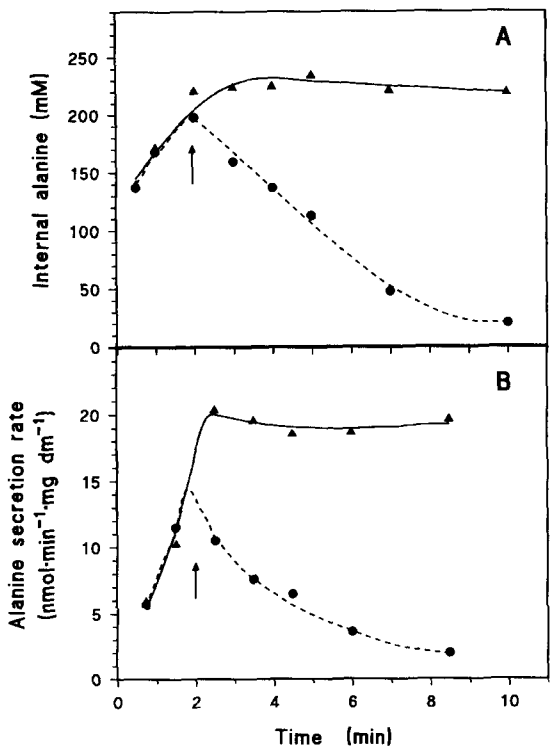

Fig. 2. Alanine fermentation in glucose-supplemented and glucose-limited cells of *Z. mobilis* CP4thi/pZY73. (A) Internal alanine concentration in cells in the presence of excess (▲) or limiting glucose (●) concentrations. The arrow indicates the time at which external glucose was zero in the case of glucose-limited cells. (B) Alanine excretion rate in glucose-supplemented and glucose-limited cells.

Since the observed rates of alanine excretion were significantly higher than the efflux rates observed in other amino acid producing microorganisms [9], the interpretation of membrane leakiness (unspecific permeability) must be taken into consideration. However, a body of evidence argues against this explanation. (i) The ATP content of *Z. mobilis* cells under alanine production conditions was 3.5–4.0 mM and thus similar to that of wild-type strains [17,26], (ii) the same held true for the electrical gradient (membrane potential), which was determined as −85 mV under alanine production conditions and for the proton motive force, which remained constant at about −135 mV. (iii) We furthermore found that uptake of glutamate, which depends on an energized plasma membrane [17], was unchanged in alanine-producing cells at about 10 nmol min^{-1} mg dry $matter^{-1}$ in the presence of 0.5 mM external glutamate.

3.1. Dependence of alanine excretion

When 100 μM of the protonophore CCCP was added to *Z. mobilis* cells under steady-state conditions of alanine excretion, both membrane potential and secondary glutamate uptake were completely abolished [17]. In contrast, the excretion rate remained unchanged (data not shown). In similar experiments we tested whether energy depletion due to lack of glucose influences alanine excretion (Fig. 2). When the external glucose was consumed, the cytosolic ATP dropped to very low levels and the membrane potential became close to zero [17], concomitantly the cytosolic alanine concentration decreased (Fig. 2). However, alanine excretion was not influenced under these circumstances and its activity closely paralleled the internal alanine concentrations. In all the experiments reported above, the amount of alanine appearing in the medium exactly corresponded to the amount of alanine disappearing in the cytosol. These experiments indicate that the sole driving force for alanine excretion in recombinant *Z. mobilis* strains is the chemical gradient of alanine.

3.2. Basic kinetic description of alanine excretion

Solute flux, solely driven by its own chemical gradient, may be explained either by simple diffusion or by a carrier-mediated uniport (facilitated diffusion) mechanism. In order to distinguish between these possibilities, we determined the kinetic parameters of alanine excretion. In the case of an efflux process, for measuring classic Michaelis–Menten kinetics, the internal substrate (alanine) concentration had to be altered. This was achieved by adding various concentrations of external ammonium (30–200 mM), in experiments similar to that reported in Fig. 1. Within the concentration range from 15 up to 280 mM internal alanine, no saturation could be observed (Fig. 3). A threshold value at low internal alanine concentrations could not be detected. The rate constant for this first-order

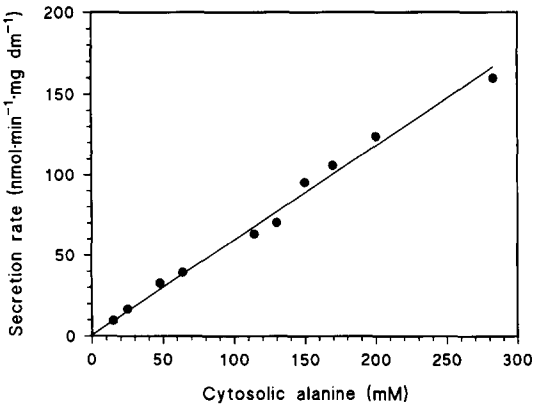

Fig. 3. Dependence of alanine excretion rate on internal alanine concentration (Michaelis–Menten plot).

process was 10^{-8} l s^{-1} mg dry matter^{-1}, or 0.28 min^{-1} for *Z. mobilis* cells under these conditions.

Besides saturation, another parameter indicating carrier-mediated excretion would be substrate specificity. In order to test this property, we passively loaded *Z. mobilis* cells with 200 mM L- or D-alanine for 2 h at 15°C. Cells were supplemented with glucose, but ammonium was omitted. After preloading, the cells were diluted 100-fold into buffer without alanine. The experiments were carried out at 15°C, in order to reduce the activity of alanine racemase. If D-alanine was used, at the end of the experiment less than 25% of the internal D-alanine was converted into the L-form. The observed excretion rate for the two stereoisomers was more or less identical, i.e. $0.33 \cdot 10^{-8}$ l s^{-1} mg dry matter^{-1} for the L-form and $0.36 \cdot 10^{-8}$ l s^{-1} mg dry matter^{-1} for the D-isomer, respectively (Fig. 4).

The third classic argument for carrier-mediated excretion would be functional inhibition. We tested a long list of different inhibitors using cells with high internal alanine concentrations in the presence of glucose and ammonium at 37°C. In order to interpret the results, possible inhibition of the glucose carrier had to be taken into account. Several different classes of reagents could be discriminated (Fig. 5). (i) SH-group reagents such as *p*-(chloromercuri)benzoate (0.2 mM), *p*-(chloromercuri)benzenesulfonate (0.2 mM) or 4-bromophenacyl bromide (0.2 mM), as well as the carboxyl reagent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.4 mM), the lysine reagent pyridoxal phosphate (10 mM), the histidine reagent diethylpyrocarbonate (0.1 mM), the arginine reagent butanedion (5 mM), and the tyrosine reagent 7-chloro-4-nitro-benzo-2-oxa-1,3-diazole (0.4 mM), neither inhibited glucose uptake nor alanine excretion. (ii) $HgCl_2$ in concentrations of up to 500 μM completely inhibited glucose uptake, although alanine excretion was unaffected. This is documented in Fig. 5 by an alanine efflux rate identical to that described above. (iii) High concentrations of *N*-ethylmaleimide ($\geq$ 2 mM) and dicyclohexyl carbodiimide (0.2 mM) slightly inhibited alanine excretion ($\leq$ 30%), however, these reagents also inhibited glucose uptake to a significant extent.

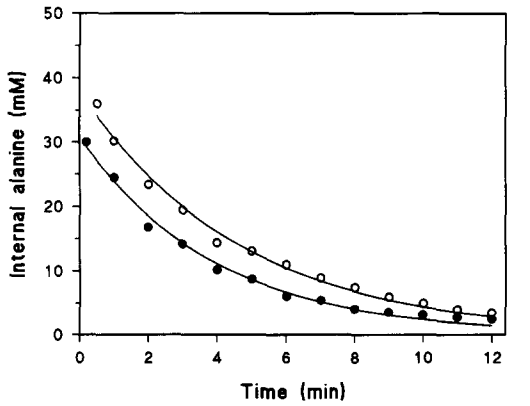

Fig. 4. Stereospecificity of alanine excretion. Cells were preloaded with L-alanine (○) or D-alanine (●). After dilution, the efflux of alanine was measured.

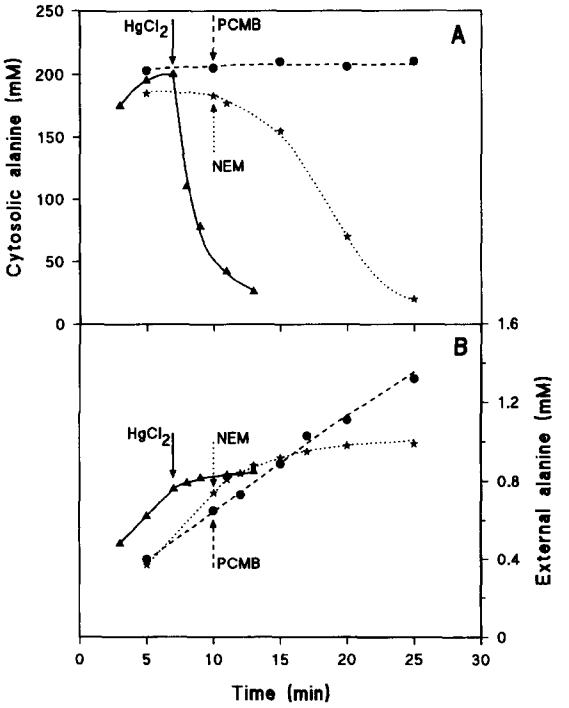

Fig. 5. Internal (A) and external (B) alanine concentrations after addition of inhibitors to alanine producing cells. Most of the inhibitors tested (see text) did not affect glucose uptake or alanine efflux, as an example, addition of 0.2 mM *p*-(chloromercuri)benzoate is shown. The effects of 0.2 mM $HgCl_2$ and 2 mM NEM on alanine excretion are explained in the text.

In contrast to channel-type activities and simple diffusion, carrier-mediated processes are in general distinguished by relatively high activation energies, due to the high energy barriers within the catalytic cycle of transport. We used *Z. mobilis* cells, incubated at 37°C in the presence of glucose and ammonium, to provide high internal alanine concentrations. The cells were then rapidly adjusted to different temperatures in the same production medium and the excretion rates were measured. Fig. 6 shows an Arrhenius plot of the observed alanine export

between 15 and 45°C. The dependence was not perfectly linear but did not show a defined break. The calculated activation energy was 29–32 kJ mol^{-1} between 15 and 45°C.

When investigating amino acid excretion in *C. glutamicum*, a valuable argument for carrier-mediated excretion, besides kinetic experiments, was provided by inactivation of the excretion carrier at high temperatures. This means that after heat inactivation in *C. glutamicum* the observed excretion rates decrease to zero (in the case of lysine [8]) or to the basic diffusion rate (in the case of isoleucine [9]). *Z. mobilis* cells, similarly treated as described above (see Fig. 6), were incubated at various temperatures between 37°C and 65°C in the presence of 200 mM external alanine in order to prevent unspecific leakage of alanine during heating. After cooling down, the cells were diluted 100-fold. Up to an incubation temperature of 55°C, no decrease in the efflux rates could be measured. Above 55°C no internal alanine pool was observed, indicating unspecific damage of the membrane (data not shown).

3.3. Measurement of counterflow maximum

A decisive result indicating the involvement of a carrier protein in the (passive) flux of solutes across a membrane is the observation of a counterflow maximum (or minimum). In order to discriminate whether alanine efflux is mediated by 'simple' or by 'facilitated' diffusion, we carried out experiments to reveal the competition between different substrates for internal binding sites, thus leading to a counterflow maximum [27,28]. In the efflux experiments at high internal alanine concentrations as described here, the external addition of labeled alanine would lead to transient internal accumulation of the label. Cells were first incubated with glucose and low or high ammonium concentrations, which led to internal alanine concentrations of 10 mM and 240 mM, respectively. After the addition of ^{14}C-labeled alanine, influx of label, although no counter-transport maximum was detected (Fig. 7). The external label simply equilibrated with internal alanine. In the case of high added ammonium, full equilibration was not reached because of the fast biosynthetic flux of (unlabeled) alanine from glucose into the cytosolic alanine pool, which concomitantly led to high excretion rates.

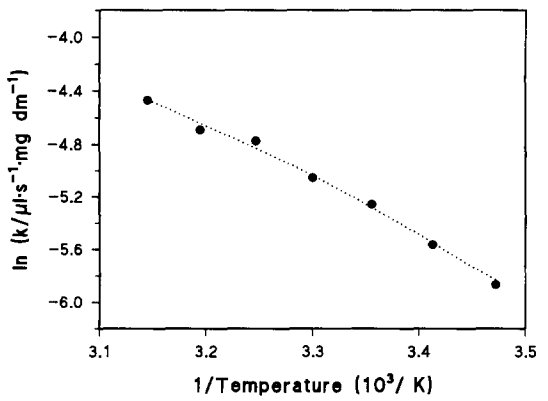

Fig. 6. Dependence of first-order rate constant of alanine excretion on temperature in the range between 15°C and 45°C (Arrhenius plot).

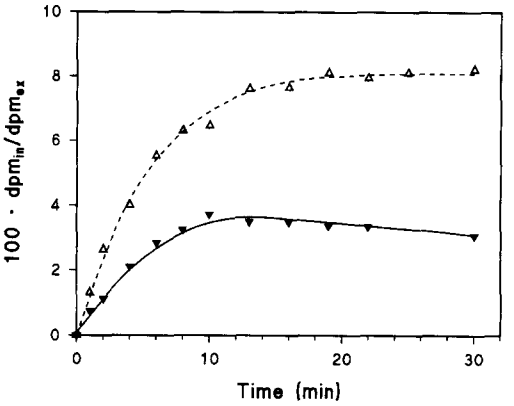

Fig. 7. Counterflow of alanine in *Z. mobilis* cells. 3 mM external alanine was added to *Z. mobilis* cells with 10 mM (▼) and 240 mM (△) internal alanine at the beginning of the experiment. The different internal alanine concentrations were established by the presence of different external ammonium concentrations (see text). The ratio of internal/external alanine in the course of the experiment is shown. The steady-state value of the internal alanine concentration, when corrected using the actual specific activity of external alanine, in fact reached 10 mM after 15 min in the case of the cells with the lower alanine concentration inside. A corresponding labeling of the internal alanine pool was not achieved in the case of cells with high internal alanine concentration due to high metabolism and consequently high efflux activity (see text). (dpm = decays per minute.)

When calculating the true influx rates of alanine by taking into account the increasing external alanine concentrations during the experiment, a rate of about 2 nmol min^{-1} mg dry $matter^{-1}$ was observed at the beginning of the experiment, which corresponded to a first-order rate constant of $0.9 \cdot 10^{-8}$ l s^{-1} mg dry $matter^{-1}$. This value is very close to the rate constant measured independently for alanine efflux (see above), and is thus in agreement with a passive, diffusion-controlled influx of alanine, too. In further experiments, we measured that influx of alanine was also nonsaturable (data not shown).

4. Discussion

Expression of the alanine dehydrogenase from *Bacillus sphaericus* in the thiamine auxotrophic *Zymomonas mobilis* strain CP4thi/pZY73 under thiamine starvation led to high cytosolic alanine concentrations and concomitantly to extremely high alanine excretion rates [5]. In principle, several different mechanisms may be responsible for amino acid excretion across the plasma membrane, and various concepts have thus been suggested. Besides (i) simple diffusion through the phospholipid bilayer, (ii) unspecific leakage caused by physical alteration due to pretreatment of the cells is conceivable. Furthermore, (iii) functional

inversion of a possible alanine uptake system could be taken into account, as well as (iv) the presence of a specific alanine excretion system. Whereas most amino acid excretion processes have been explained in the past by concepts (i) and (ii) [9,29], clear evidence for the presence of specific amino acid excretion systems in *Corynebacteria* has been obtained recently [6–9].

We did not observe experimental evidence for the function of an alanine excretion system in *Z. mobilis* (concept iv), neither could we detect carrier-mediated alanine uptake, which would be required for concept (iii), i.e. functional inversion of an uptake system. On the contrary, also alanine uptake appeared to be a insatiable, diffusion-controlled process. Alanine excretion was not dependent on cytosolic ATP or on the electrochemical potential, the only driving force for alanine excretion was its chemical gradient across the plasma membrane. All classic arguments for carrier-mediated transport, i.e. saturation, substrate specificity (stereospecificity) and inhibition were absent. Most convincingly, we showed that during alanine flux across the membrane, no countertransport maximum occurred. In accordance with this interpretation, the activation energy of alanine excretion was relatively low.

Since, on the other hand, the observed alanine excretion rates were extraordinarily high, the possibility of membrane damage as a result of the conditions of cell pretreatment, i.e. long starvation period of the thiamine-deficient cells under high external ammonium acetate concentrations, had to be considered. It has been shown previously that *Z. mobilis* is very sensitive to energy depletion [17]. However, we found that the plasma membrane and the energetic conditions of the cell remained intact. We did not observe unspecific leakage of other compounds, on the contrary, secondary uptake of glutamate from the surrounding medium was still active, leading to a high steady state accumulation ratio. Cytosolic ATP levels were similar to the wild type under growth conditions. The reduced membrane potential (-85 mV instead of -120 mV in the wild type under these conditions) could be experimentally correlated to the presence of the high concentration of ammonium acetate, which acts as an uncoupling agent at pH 7.0. A similar reduction could be observed in the wild-type strain in the presence of high ammonium concentrations (data not shown).

Thus, the only explanation for alanine excretion in the recombinant *Z. mobilis* strain CP4thi/pZY73 under thiamine starvation, which agrees with the experimental data, is simple diffusion across the plasma membrane, characterized by an extraordinarily high diffusion rate constant of 10^{-8} l s^{-1} mg dry $matter^{-1}$, or 0.28 min^{-1}. It is interesting to compare this finding with data from other organisms. In contrast to former interpretations, in *C. glutamicum*, a well-known amino acid producing bacterium, specific excretion systems have been found to be present for at least lysine [8], glutamic acid [30], and isoleucine [6]. The diffusion constant for isoleucine has been determined

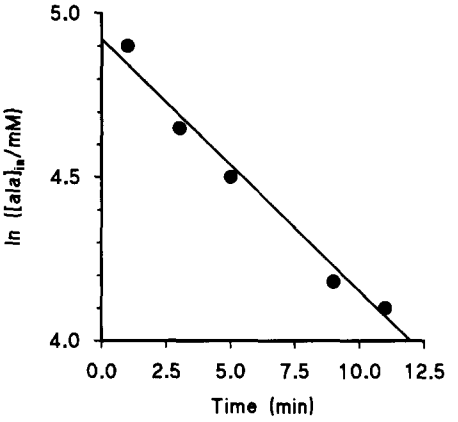

Fig. 8. Alanine excretion by preloaded *C. glutamicum* cells. *C. glutamicum* cells were preloaded with alanine by peptide feeding. After depletion of the dipeptide, the efflux of alanine in resting cells was measured.

as $0.21 \cdot 10^{-8}$ l s^{-1} mg dry $matter^{-1}$, or 0.08 min^{-1} in *C. glutamicum* [9]. Isoleucine is clearly more hydrophobic than alanine. Consequently the diffusion constants for alanine across bacterial membranes which have been published as 0.03 min^{-1} [31] or about $5 \cdot 10^{-10}$ l s^{-1} mg dry $matter^{-1}$ (calculated from [29] and [10] where the diffusion of proline was measured and compared with alanine) are lower than both the value we measured for isoleucine in *C. glutamicum* and especially the value observed for alanine in *Z. mobilis*. Lower rates of isoleucine diffusion have also been reported for *Clostridium saccharoperbutylacetonicum* [13]. However, since the internal alanine concentration was not measured, a diffusion rate cannot be calculated. Thus, in order to correlate our findings to data in the literature, we determined the alanine diffusion rate in *C. glutamicum* (Fig. 8). In analyzing the decrease of alanine accumulated in resting *C. glutamicum* cells under deenergized conditions, we measured a first-order rate constant of excretion of $0.2 \cdot 10^{-8}$ l s^{-1} mg dry $matter^{-1}$. We did not find any evidence for the involvement of a carrier in this process in various control experiments (data not shown). Thus, alanine diffusion in *C. glutamicum* is significantly slower than the same process observed in *Z. mobilis*. Since *Z. mobilis* did not show increased permeability of other solutes, the high diffusion rate of alanine must be a particular property of *Z. mobilis* cells. It is important to note that the plasma membrane of this organism is clearly different from most other bacteria in containing high amounts of hopanoids (bacterial triterpenes or steroid equivalents) [32,33]. This may be responsible for the particular permeability properties. Experiments to elucidate this situation by using artificial membranes are in progress.

Acknowledgements

We thank H. Sahm for continuous support and helpful discussions. We are indebted to S. Zittrich for measure-

ment of alanine diffusion in *C. glutamicum* and to C. Lambert for technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Kr 693/5) and by the Fonds der Chemischen Industrie.

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