Dissection of Ammonium Uptake Systems in *Corynebacterium glutamicum*: Mechanism of Action and Energetics of AmtA and AmtB

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Ammonium uptake systems have been described in many bacteria, although its uncharged form, ammonia (NH₃), is supposed to be highly membrane permeable. Until 1998, exclusively energy-dependent, membrane potential-driven ammonium transport was proposed, when Soupene and coworkers argued that AmtB proteins in enteric bacteria increase the rate of equilibration of uncharged ammonia across the cytoplasmic membrane rather than actively transporting and accumulating the solute.

In *Corynebacterium glutamicum*, two different ammonium transporters are present. AmtA exhibits a relatively high affinity of 44 ± 7 μM for methylammonium, an ammonium analogue commonly used for transport measurements (5, 12, 18, 19), and a maximal velocity of 25 ± 5 nmol mg (dry weight)⁻¹ min⁻¹. Uptake is dependent on the membrane potential and can be abolished by the addition of protonophores, like carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (18). In the presence of 50 μM methylammonium, 10 μM of ammonium is sufficient for a half-maximal inhibition of methylammonium uptake, indicating a high affinity of AmtA for this solute (12). In contrast to AmtA, no methylammonium uptake activity was detected for AmtB until now (12).

The aim of this study was to investigate the contributions of AmtA and AmtB to methylammonium/ammonium transport and to address the question of the transport mechanism(s) and energetic coupling.

**Construction of mutant strains.** To investigate whether metabolic trapping by GS is a major driving force for methylammonium/ammonium uptake via Amt proteins, *glnA2* coding for a GS without known function (15), and *gdh* coding for glutamate dehydrogenase (GDH)—in addition to *glnA*, encoding the only active GS in *C. glutamicum*—were deleted as described previously (17). The triple-deletion strain DA-2 (*ΔglnA ΔglnA2 Δgdh*) was generated based on strain TMΔgdhΔglnA (13) using plasmid pK18ΔglnA2 (15), which carries an internal *glnA2* deletion. Plasmid pKΔamtB, carrying an internal *amtB* deletion (14), was used to generate a *glnA glnA2 gdh amtB* quadruple-mutant strain, MeK-1, based on strain DA-2. The resulting strains were tested with respect to ammonium assimilation and nitrogen control (data not shown). Transcription and Western blot analyses were carried out using probes and antisera directed against GS and GDH. As expected, the deletion strain DA-2 and MeK-1 lacked the corresponding mRNAs and proteins, and neither GS nor GDH activity was detectable (data not shown). Furthermore, polar effects of the introduced mutations were excluded by complementation assays and transcription analyses (data not shown).

To investigate the effect of the absence of methylammonium/ammonium assimilation in strain DA-2, cell extracts were prepared from cells incubated with methylammonium and subjected to thin-layer chromatography (12, 19). While in the wild-type ATCC 13032 (1) methylammonium was completely converted by GS to an unidentified assimilation product, most likely methylglutamine, as discussed previously (12), only free methylammonium was detectable in the *glnA glnA2 gdh* mutant DA-2 (Fig. 1).

**Differentiation of methylammonium fluxes.** To discriminate the three possible methylammonium uptake pathways, i.e., uptake via AmtA and AmtB, respectively, as well as influx by passive diffusion, the uptake rates depending on the external methylammonium concentration for the wild-type ATCC 13032 (1), the *amtB* mutant LN-1.1 (12), the *amtA* mutant MJ2-38 (12), and the *amtA*/*amtB* mutant JS-1 (20) were measured as described previously (12, 18) (Fig. 2). As indicated by the comparison of the wild type, LN-1.1, and MJ2-38, AmtA is the major uptake system for methylammonium in *C. glutamicum*, while AmtB contributes significantly to the total uptake only at very high external substrate concentrations. This is also the reason why in previous publications, where uptake was measured at methylammonium concentrations not exceeding...
Within the range of methylammonium concentrations tested here, AmtB-mediated uptake was linearly dependent on the external substrate concentration and was not saturable up to 3 mM of external methylammonium. In the \textit{amtA amtB} mutant JS-1, methylammonium transport was negligible. It should be taken into consideration that JS-1 still harbors the ammonium-assimilating enzymes, which means that metabolic trapping is in principle fully functional. Consequently, the contribution of passive diffusion to the total methylammonium flux in the substrate concentration range used for kinetic measurements presented in the following experiments is less than 1%.

**Energetics of methylammonium uptake.** Metabolic trapping of ammonium or methylammonium by GS activity was always a major argument in connection with ammonium uptake in bacteria (5, 19). When methylammonium uptake rates mediated by AmtA depending on the substrate concentration in strain MeK-1, which lacks GS, GDH, and AmtB, were measured in comparison with those in strain LN-1.1, which still carries the ammonium-processing enzymes, the two strains were not significantly different in terms of uptake kinetics (Fig. 3). Consequently, the mechanism of metabolic trapping is not valid for ammonium/methylammonium uptake by AmtA.

As an alternative driving force, we tested the electrochemical potential across the plasma membrane. The influence of the membrane potential can be quantitated with respect to both kinetic (uptake rates) and thermodynamic (accumulation ratio) values. First, we measured the methylammonium uptake kinetics within 3 min after addition of the labeled substrate (Fig. 4). In order to identify a possible dependence of this uptake rate on the membrane potential, we decreased the electrical gradient by increasing the power of uncoupling reagents, using the addition of a low concentration of CCCP (3 μM), leading to partial uncoupling only, as well as a high concentration of CCCP (50 μM). As a control, the same experiment was carried out using the \textit{amtA amtB} deletion strain JS-1. Background values for cell and filter binding were subtracted. These were determined by carrying out experiments in the presence of 0.1% (wt/vol) of the cationic detergent cetyltrimethylammonium bromide (CTAB), which permeabilizes the cell membrane without fully disrupting the cells (16). The initial uptake rates calculated from Fig. 4 result in about 10, 4, and 2 nmol mg (dry weight)\(^{-1}\) min\(^{-1}\) for the different conditions applied to the deletion strain MeK-1 (no addition, 3 μM, and 50 μM CCCP, respectively). The initial uptake rates calculated from Fig. 4 result in about 10, 4, and 2 nmol mg (dry weight)\(^{-1}\) min\(^{-1}\) for the different conditions applied to the deletion strain MeK-1.

Since substrate accumulation, which, in contrast to uptake
rates, is a thermodynamic value, might be more convincing for defining energetic aspects of transport, experiments were carried out to determine the chemical gradient (accumulation ratio) of methylammonium (internal/external concentration) under steady-state conditions, i.e., after long-term uptake. These experiments were only possible based on the availability of strain MeK-1, because the putative driving force (membrane potential) is present while metabolic trapping is absent and, equally important, because the passive diffusion of methylammonium is negligibly low in *C. glutamicum* under the experimental conditions used. For a correct calculation of accumulation ratios, the supernatant (external methylammonium) and cells (internal methylammonium) were harvested separately and the substrate concentration was quantified (Fig. 5). The cells accumulated methylammonium to different extents, and a situation of approximate steady state was reached after about 10 min of uptake. Untreated cells of strain MeK-1, i.e., devoid of metabolic trapping and lacking the second uptake system, AmtB, achieved a steady-state accumulation ratio of up to 23,000 (internal/external), whereas the steady-state accumulation was significantly decreased by the addition of uncouplers. Strain JS-1, devoid of both uptake systems but equipped with enzymes for ammonium metabolism and thus capable of metabolic trapping, was characterized by very low methylammonium accumulation, most probably due to adsorption. As a control, we also measured the methylammonium accumulation in cells permeabilized by low concentrations of CTAB, which indicated a residual amount of adsorption of transport substrate.

**Concluding remarks.** The dissection of methylammonium fluxes achieved in this work in *C. glutamicum* cells demonstrates that (i) the function of AmtA is fundamentally different from that of AmtB, (ii) AmtA is the predominant ammonium uptake system in *C. glutamicum*, (iii) uptake of methylammonium via AmtA is independent of the possible driving force of metabolic trapping and thus represents an energy-driven uptake mechanism, and (iv) uptake of methylammonium is dependent on the presence of a membrane potential in terms of both the uptake rate and steady-state accumulation. The dependence of uptake rates on the membrane potential has been observed before in several cells, including *C. glutamicum* (references 6, 12, and 18 and references therein). In the experiments on steady-state methylammonium accumulation, we observed extremely high values, which, however, were substantiated by appropriate controls using cells devoid of ammonium uptake systems, as well as permeabilized cells. Furthermore, the observed high steady-state accumulation of methylammonium in the cytoplasm in the absence of any significant pH gradient again argues for the membrane potential being the driving force for this energy-consuming process. This conclusion directly argues for a net positive charge(s) being moved in the course of methylammonium uptake in *C. glutamicum*, which is not in line with transport of uncharged ammonia. Very recently, Fong and coworkers (4) provided experimental evidence that, in contrast to their previous hypothesis (19), in fact the charged methylammonium seemed to be the species transported by *E. coli* AmtB. In principle, this is in line with the experimental data presented here. However, there is an obvious difference between the two investigations, due to the low permeability of *C. glutamicum* membranes to methylammonium. We were able to quantify the passive (background) flux of methylammonium by using a strain devoid of both the *amtA* and the *amtB* genes but still harboring the metabolizing enzymes and thus the system of metabolic trapping.

The high accumulation ratio of up to 23,000 (internal/external methylammonium concentration) observed in our experiments using recombinant *C. glutamicum* strains is surprising. It is not in line with a basic conception of the methylammonium cation being the sole transport substrate of *C. glutamicum*...
AmtA. Steady-state accumulation values higher than 500, which would equilibrate the typical membrane potential of 160 mV measured in *C. glutamicum* under the growth conditions used (unpublished results), indicate the transfer of more than one charge in the course of substrate translocation by AmtA. This will be investigated in more detail in future studies.

In view of its very low activity compared to AmtA, AmtB does not seem to play a significant role in physiological conditions. The mechanism of transport by AmtB is less clear, but it is different from that of AmtA. The low accumulation of methylammonium by AmtB in the absence of metabolic trapping would in principle be in agreement with a pore-like mechanism accepting the uncharged species as the substrate. The affinity of AmtB for methylammonium seems to be extremely low, since we did not achieve saturation within a concentration range of up to 3 mM. Consequently, characterization of AmtB in the AmtA deletion mutant was hampered by this fact, which leads to very low transport activity at reasonable concentrations of labeled substrate. In any case, the observed results do not argue for an electrophoretic mechanism of AmtB, nor are they in obvious agreement with the kinetic behavior of AmtB proteins in other organisms.

It was shown earlier by Ludewig, Mayer, and coworkers that Amt proteins in plants seem to function as ammonium transporters (8, 10, 11), while the closely related animal Rh proteins function as ammonia channels. This was taken as an indication that related proteins with high structural similarity can function according to different mechanisms (9). In fact, *C. glutamicum* AmtA has a very high degree of similarity to other bacterial Amt proteins, including AmtB, with 68% identical amino acids. The two strategic phenylalanine residues at the entry into the substrate pathway are present in AmtA from *C. glutamicum*, as well as the highly conserved two histidine residues in the central part of the pathway (6). Consequently, based on the primary structure, there is no indication of a different function for *C. glutamicum* AmtA compared to other Amt proteins in bacteria.

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