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EVIDENCE FOR AMMONIA TRANSLOCATION BY CLOSTRIDIUM PASTEURIANUM

D. Kleiner and E. Fitzke

Chemisches Laboratorium der Universität Freiburg, Albertstrasse 21, 7800 Freiburg, West-Germany

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ABSTRACT: Clostridium pasteurianum is able to take up NH_4^+ and $CH_3NH_3^+$ against concentration gradients. Uptake of $CH_3NH_3^+$ is abolished by NH_4^+ and partially inhibited by dinitrophenol. C. pasteurianum membranes are permeabilized for NH_4^+ by valinomycin. These results are regarded as evidence for an ammonium translocase in membranes otherwise only slightly permeable for NH_3.

INTRODUCTION: Fixation of N_2 is a very energy consuming process (see ref. 1). Thus organisms capable of N_2 assimilation usually prefer other nitrogen sources over N_2, and in their presence repress the formation of nitrogenase. The most preferred nitrogen sources known are ammonium salts. N_2 fixing organisms such as Rhodospirillum rubrum, Azotobacter vinelandii, Klebsiella pneumoniae or Clostridium pasteurianum are able to efficiently scavenge their environments for traces down below 1 μM of this compound (2 - 6). Here we wish to present evidence, that this ammonium uptake by C. pasteurianum is an energy dependent translocation process against a concentration gradient. This conclusion is based on three lines of experimental results: a) C. pasteurianum membranes are rather impermeable to ammonia, b) NH_4^+ concentration gradients across these membranes exist, c) $^{14}CH_3NH_3^+$ is absorbed concentratively, and this uptake is inhibited by NH_4^+ salts and dinitrophenol.

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MATERIALS and METHODS: C. pasteurianum W5 was grown routinely in a continuous culture (dilution rate 0.25, pH 6.4, 30°C) with N_2 and sucrose as nitrogen and carbon source. Bacterial growth was followed spectrophotometrically at 660 nm. Nitrogenase activities in vivo were determined by the acetylene reduction technique under N_2 as described before (3). Ammonia was assayed with an ammonia electrode. Valinomycin was obtained from Sigma, St. Louis, USA, $^{14}CH_3NH_3Cl$ from Rohstoff-Einfuhr GmbH, Düsseldorf, FRG.

RESULTS: Evidence for C. pasteurianum membranes being rather impermeable to NH_4^+.

C. pasteurianum is able to reduce N_2 to NH_4^+ and to keep the product within the cellular envelope without major diffusion to the outside (6). This can be explained either by a fast metabolization of NH_4^+, thus keeping the intracellular concentration very low or by impermeability of the plasma membrane to NH_3. Evidence for the second possibility was provided by the fact that leakage of NH_4^+ could be induced by addition of the specific ionophore valinomycin. Since valinomycin permeabilizes the membranes for both NH_4^+ and K^+, and since growth on several carbon sources requires the presence of a K^+ gradient, a 500 ml batch culture of C. pasteurianum was grown on fructose with solid $CaCO_3$ as buffering agent, 10 mM KH_2PO_4 and micronutrients as described before (7). Uptake of fructose is mediated by a phosphotransferase system (8) and thus does not require a K^+ gradient. Fig. 1 shows that addition of valinomycin results in NH_4^+ excretion, reduction of nitrogenase activity and a reduced growth rate. The rate of NH_4^+ excretion depends on the amount of valinomycin added: by in-

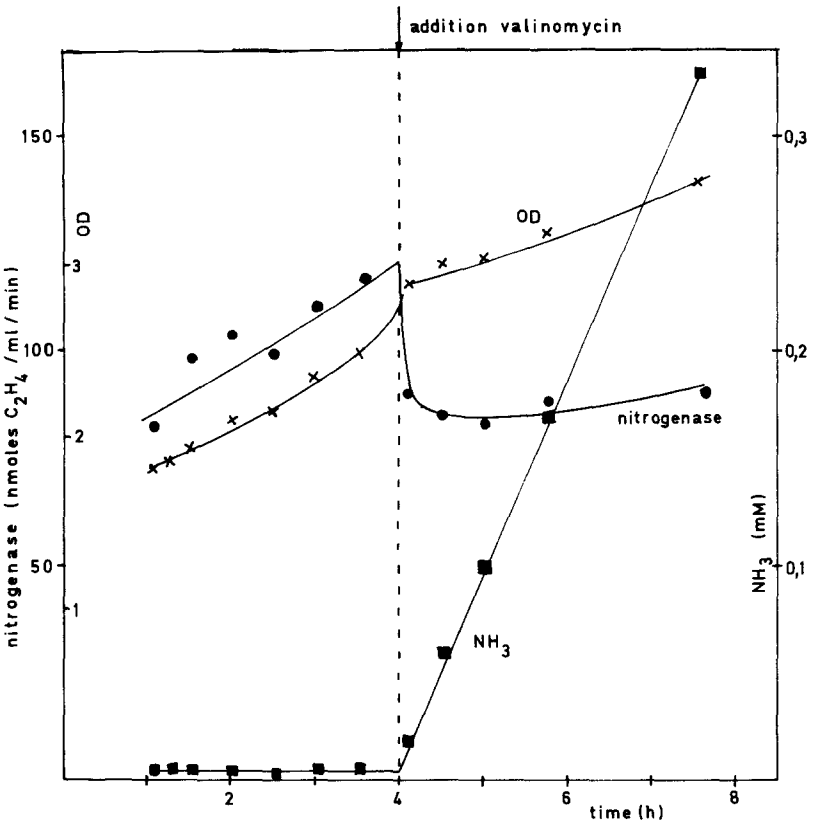

Fig. 1. Appearance of extracellular NH_4^+ (■), changes in nitrogenase activity (●) and bacterial density (x) after addition of 5 mg valinomycin to a 500 ml batch culture of C. pasteurianum.

creasing the dose threefold excretions up to 1 mM within 2 h could be obtained.

Determination of NH_4^+ gradients across the plasma membrane.

Extracellular NH_4^+ concentrations were determined after removal of the organisms by centrifugation (3000 x g, 10 min). For the determination of intracellular NH_4^+ samples of the culture were immediately treated with NaOH to a final concentration of 1 M. Upon this treatment the cells disintegrate and the NH_3 liberated can be immediately measured with the ammonia electrode. Intra-

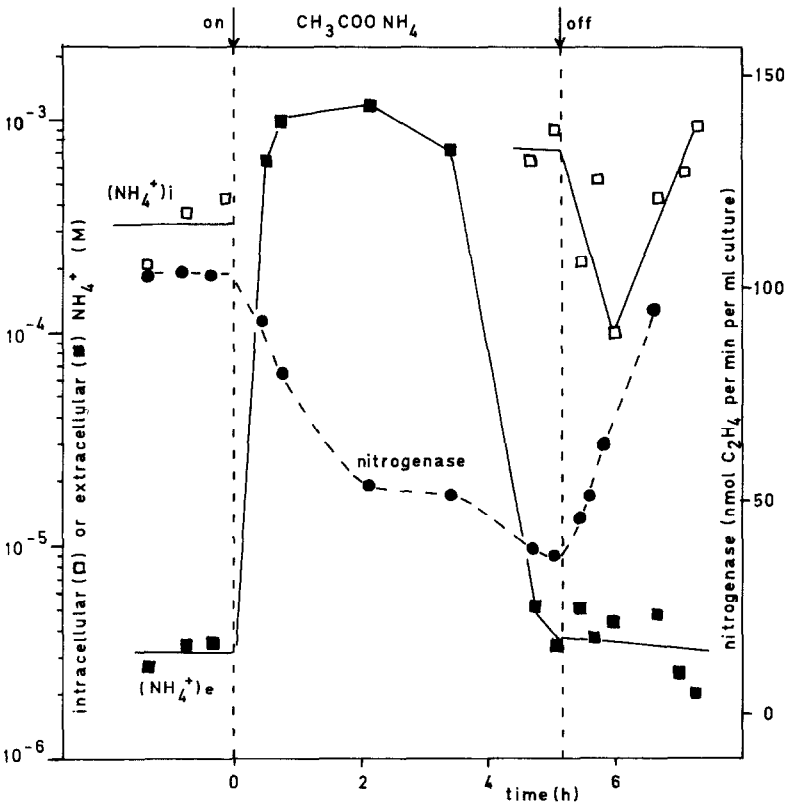

Fig. 2. Changes of the intracellular (□) and extracellular (■) NH_4^+ concentration and nitrogenase activity (●) before and after continuous addition of 1.5 mmol/h CH_3COONH_4 to a 1.2 l continuous culture of C. pasteurianum.

cellular concentrations then were calculated by dividing the difference by five times the dry weight of the sample. With this method consistently about 100 fold gradients were found. This amounted under N_2 fixing conditions to intracellular NH_4^+ levels of about 0.2 mM, in the same range as found previously for A. vinelandii (4) and K. pneumoniae (5). If small amounts of NH_4^+ salts are added continuously to the continuous culture, the extracellular NH_4^+ level rises (Fig. 2), while nitrogenase synthesis is repressed. (Under these circumstances, determinations of intracellular NH_4^+ was impossible.) When the

Table 1

Concentrative uptake of $^{14}CH_3NH_3^+$ by C. pasteurianum

Addition	$\frac{\text{intracellular}}{\text{extracellular}}$ $^{14}CH_3NH_3^+$
none (residual NH_3 conc. in culture 2 μM)	71
0.1 mM dinitrophenol	49
0.5 mM CH_3COONH_4	3.7
0.1 mM CH_3COONH_4	3.8
0.02 mM CH_3COONH_4	5.3
incubation time: 1 min	

nitrogenase level falls below a certain value, all incoming NH_4^+ is absorbed immediately against a NH_4^+ gradient. When the NH_4^+ influx ceases, a sharp drop in the intracellular NH_4^+ level is observed. Upon resumption of nitrogenase synthesis this level rises again.

Control experiments with glutamine and carbamyl phosphate showed that under these conditions neither compound liberated NH_3. Thus hydrolysis of these substances as sources of intracellular ammonia is highly unlikely.

Concentrative uptake of methylamine

Concentrative uptake of $^{14}CH_3NH_3^+$ and (competitive) inhibition by NH_4^+ have been repeatedly used as indication of NH_4^+ translocation (9 - 13). For the present studies 2 ml samples were withdrawn from the continuous culture and incubated with 15 μM $^{14}CH_3NH_3Cl$ for various lengths of time and with various additions. After fast centrifugation (15 s) the pellets were suspended in 1 N $HClO_4$, extracted for 10 min, centrifugated again, and the radioactivity in both supernatants was compared. Table 1 shows that a) at least a 70-fold gradient of methylamine

can be established across the membrane, b) dinitrophenol partially inhibits gradient formation, c) NH_4^+ almost completely abolishes $^{14}CH_3NH_3^+$ uptake. Dinitrophenol disturbs the energy metabolism in this organism and causes breakdown of a pH gradient across the plasma membrane (14). Methylamine gradients were established within less than 30 s, thus uptake kinetics could not be measured with our technique. The gradients were maintained for various lengths of time. Usually after 2 to 10 min leakage of radioactivity occurred for reasons unknown to us.

In order to show that the intracellular $^{14}CH_3NH_3^+$ was not metabolized immediately, the radioactive extracts were chromatographed on Dowex 1 x 8, a strong anion exchange resin. All the radioactivity from cells which had been incubated less than 2 min with $^{14}CH_3NH_3^+$ could be eluted from the resin at pH $\geq$8, indicating presence of ^{14}C in a cation or uncharged molecule. This was regarded as evidence for no immediate metabolization of CH_3NH_2. After prolonged incubation up to 30% of the radioactivity could only be eluted with 0.1 M HCl, indicating slow metabolization by the organisms.

DISCUSSION: It has long been assumed that the uncharged molecules NH_3 and CH_3NH_2 are able to penetrete passively through most if not all biological membranes (see 15). However, since 1970 in a number of eukaryontes (9 - 12) and recently also in *E. coli* (13) concentrative uptake of $^{14}CH_3NH_2$ has been demonstrated and regarded as evidence for a NH_4^+ translocase. As has been pointed out by Stevenson and Silver (13), the formation of a specific NH_4^+ transport system would be of selective advantage under nitrogen-limited growth, since under physiological conditions more than 99% of ammonia is present

as NH_4^+. The existence of NH_4^+ translocase implies considerable impermeability of the cytoplasmic membrane towards NH_3. This impermeability must be of necessity for organisms fixing N_2, since otherwise a considerable part of the ammonia formed from N_2 would escape from the cytoplasm. This leakage should be enhanced by a pH-gradient with a higher pH inside, the existence of which has been demonstrated for C. pasteurianum (14). In line with these reasonings the evidence presented here indicates that the membrane of C. pasteurianum is rather impermeable towards ammonia, that NH_4^+ gradients are maintained during growth, and that concentrative uptake against these gradients is possible.

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