Energy expenditure for cyclic retention of NH_3/NH_4^+ during N_2 fixation by *Klebsiella pneumoniae*

D. Kleiner

Lehrstuhl für Mikrobiologie, Universität Bayreuth, Postfach 3008, 8580 Bayreuth, FRG

Received 14 May 1985

The permeability coefficeint for NH_3 through bacterial membranes was determined to be around 2×10^{-3} $cm \cdot s^{-1}$. This value was used to calculate the outward diffusion of NH_3 from intracellular pools and the energy costs for reabsorption as NH_4^+. For an N_2-fixing continuous culture of *Klebsiella pneumoniae* an energy expenditure of around 4 ATP per NH_3 produced was calculated, thus increasing significantly the energy requirement for N_2 fixation in vivo.

NH_3 cyclic retention *NH_3 permeability* *N_2 fixation energetics*

1. INTRODUCTION

When grown with nitrogen sources other than NH_4^+ most bacteria derepress specific NH_4^+ transport systems, which are postulated to be responsible for recovery of NH_4^+ lost by diffusion as NH_3 from an internal pool [1–3]. This hypothesis is based on the observations, that: (a) many bacteria repress the NH_4^+ carriers during growth on NH_4^+; then diffusion of NH_3 must be fast enough to support the cell's demand for nitrogen; (b) mutants lacking NH_4^+ carriers constantly excrete NH_3 [3]. These results indicate considerable permeability of bacterial membranes towards NH_3. Here, I report the quantification of NH_3 movements across a bacterial membrane, and an estimation of the energy expenditure for cyclic NH_3/NH_4^+ retention in an N_2-fixing culture of *Klebsiella pneumoniae*.

2. MATERIALS AND METHODS

K. pneumoniae M5a1 (a gift from Professor R.H. Burris, University of Wisconsin, Madison) was either grown anaerobically with N_2 or aerobically with NH_4^+ under sulfate (0.04 mM) limiting conditions in continuous culture [4]. The mutant KP5060 [5] was grown in 20 ml batches in the same minimal medium containing 100 mM phosphate, and 2 mM histidine, 2 mM glutamine and 20 mM aspartate as nitrogen sources. [^{14}C]Methylammonium uptake as an indicator of NH_4^+ transport was measured as in [2]. Intracellular NH_4^+ was extracted by a modification of Gerth's method [6]: 8 ml culture were centrifuged through a layer of silicone oil into 1 ml extraction mixture containing 1% phenol, 1% toluene, 10% Tween 80 and 5% KCl in H_2O. NH_4^+ was estimated by the method of Fawcett and Scott [7] after micro-diffusion. Total fixed nitrogen was estimated by Kjeldahl digestion of culture aliquots.

3. RESULTS

N_2-fixing cells of *K. pneumoniae* lose some of their newly produced NH_3 through the membrane [1,3]. To estimate the energy expenditure for NH_4^+ reabsorption, the ratio of NH_3 diffusion to NH_3 assimilation was determined. NH_3 assimilation can be estimated from increase in the total nitrogen content of a culture. NH_3 diffusion in the steady state is governed by Fick's First Law [8]:

$$\frac{dn}{dt} = -PA\Delta c \qquad (1)$$

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30 © 1985 Federation of European Biochemical Societies

where dn/dt is the number (mol) of solute molecules traversing a membrane with area A per unit time, Δc is the NH_3 concentration difference and P the permeability coefficient. The pertinent values were determined for an N_2-fixing continuous culture of *K. pneumoniae* (pH 6.6; dilution rate (D) 0.2 h^{-1}), which at 30°C contained 2.7 $\times$ 10^9 bacteria/ml, as determined by appropriate dilution and plate counting. Microscopic inspection gave an average cell dimension of 1.5 $\times$ 0.75 μm, resulting in a total bacterial surface A of 120 cm^2 and a total bacterial volume V of 1.8 $\times$ 10^{-3} cm^3/ml culture. Intracellular NH_4^+ was determined as 1.0 $\pm$ 0.35 nmol/ml culture, yielding an intracellular NH_4^+ level of 0.56 $\pm$ 0.25 mM at an extracellular concentration of 15 μM. Assuming an intracellular pH of 7.4 [9] and taking into account a pK = 9.25 for the protonation of NH_3, $\Delta c(NH_3)$ was calculated to be 8 μM.

For the estimation of the permeability coefficient P 2 methods were employed. First an aerobic sulfate limited continuous culture was set up (pH 6.9, D = 0.22, 1.9 $\times$ 10^9 cells/ml at the steady state). By decreasing the extracellular NH_4^+ content and monitoring the [^{14}C]methylammonium transport rate, a distinct external NH_4^+ level of 2.5 mM was determined, at which the NH_4^+ carrier just became derepressed. The inflowing medium contained 5.5 mM. At this point inward NH_3 diffusion was just sufficient for the nitrogen supply. From the dilution rate and the disappearance of NH_4^+ from the medium a diffusion rate dn/dt = 0.66 μmol/h per ml culture was calculated. Since the intracellular NH_4^+ level could not be measured under these conditions (adherence of extracellular NH_4^+ at the cell), $\Delta c(NH_3)$ was calculated by assuming the intracellular NH_4^+ level to be the same as under N_2-fixing conditions (0.56 mM). Assuming an intracellular pH of 7.5 [9] at the extracellular pH of 6.9, a $\Delta c(NH_3)$ of 0.0012 μmol/cm^3 was calculated (intracellular [NH_3] = 10.0 μM; extracellular [NH_3] = 11.2 μM). Inserting the values for dn/dt and Δc, and a total bacterial surface A = 84 cm^2/ml culture into eqn 1, $P(NH_3)$ was determined to be

$$P(NH_3) = \frac{0.66}{0.0012 \times 84 \times 3600} = 1.8 \times 10^{-3} \text{ cm/s}$$

Since $\Delta c(NH_3)$ is the difference of large numbers, and since both the intracellular pH and the periplasmic pH are known only approximately, the calculated P value contains a wide margin of error. For support the permeability coefficient for methylamine P(MA) was determined by a different method. The mutant KP5060, which is deficient in both glutamine synthetase [5] and in ammonium transport [2] was incubated with 1.0 mM [^{14}C]methylammonium for 1 h at pH 7.5. Then the cells were rapidly diluted into a 100-fold excess of the same buffer containing 1 mM unlabelled methylammonium, and the decrease of the intracellular radioactivity was determined. As shown in fig.1, efflux followed first-order kinetics, was very fast and strongly temperature dependent (apparent activation energy around 35 kJ/mol). Assuming intra- and extracellular pH are the same (7.5), P can be calculated from the transformed equation

$$P = -\frac{1}{\Delta c} \cdot \frac{dc}{dt} \cdot \frac{V}{A} \qquad (2)$$

with $-dc/dt$ being the decrease in the intracellular

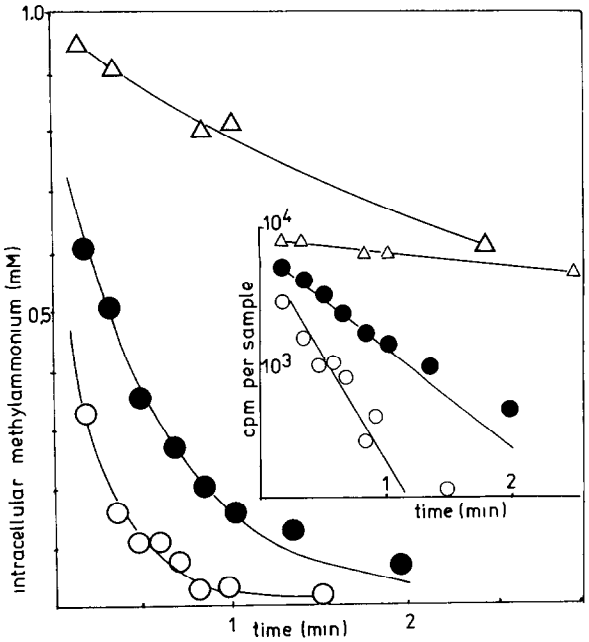

Fig.1. Efflux of [^{14}C]methylamine from pre-loaded cells of mutant KP5060 at (○) 30°C, (●) 18°C, (△) 0°C. Residual content at equilibrium (t = 1 h) was subtracted. Inset: raw data in a semi-logarithmic plot.

methylammonium concentration per s, Δc the methylamine concentration difference at a certain moment, and V/A the volume to surface ratio of a bacterium. Taking into account a $pK_a = 10.7$, a value of $P(MA) = 1.5 \times 10^{-3}$ cm/s was obtained from the data. This value is in good agreement with $P(NH_3) = 1.8 \times 10^{-3}$ cm/s. Interestingly, the values are in the same range as $P(H_2O)$ for bilayer lipid membranes [8].

Using $P(NH_3) = 1.8 \times 10^{-3}$ cm/s for the calculation of NH_3 leakage in the continuous N_2-fixing culture, the diffusion rate according to eqn 1 amounted to

$$\frac{dn}{dt} = 1.8 \times 10^{-3} \times 120 \times 8 \times 10^{-9} = 1.7 \times 10^{-9}$$

mol NH_3 lost/s per ml culture from the bacteria. From Kjeldahl digestion a nitrogen content of 0.07 mg/ml culture was calculated. Thus 0.28 nmol N were assimilated/s per ml of the same culture at $D = 0.2\ h^{-1}$.

4. DISCUSSION

For the culture employed, disappearance of NH_3 by diffusion was about 6-times faster than by glutamine formation. This implies that each NH_3 molecule produced by N_2 fixation, on average, passes the retention cycle about 6 times before being assimilated. Each cycle involves the net influx of one H^+, which under the anaerobic conditions has to be expelled by the H^+ translocating ATPase. If an average H^+/ATP stoichiometry of 3 is taken as for *E. coli* [10], the pathway from NH_3 to glutamine under the conditions employed costs 2 ATP for the cyclic retention of each molecule NH_3 or 4 ATP per N_2 molecule fixed. This biologically significant energy expenditure for the maintenance of an internal NH_3 pool may partially resolve a long-standing problem in energetics of N_2 fixation. Generally, cell-free nitrogenase preparations consume some 16 mol ATP per mol N_2 fixed. However, from growth yield determinations, whole cells of *K. pneumoniae* were shown to require 29 ATP per N_2 fixed [11]. Part of the additional energy is used for glutamine synthesis, but a greater part probably for cyclic NH_3/NH_4^+ retention.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Kleiner, D. (1985) FEMS Microbiol. Rev. 32, in press.
[2] Kleiner, D. (1982) Biochim. Biophys. Acta 688, 702–708.
[3] Castorph, H. and Kleiner, D. (1984) Arch. Microbiol. 139, 245–247.
[4] Kleiner, D. (1976) Arch. Microbiol. 111, 85–91.
[5] Streicher, S.L., Shanmugam, K.T., Ausubel, F., Morandi, C. and Goldberg, R.B. (1974) J. Bacteriol. 120, 815–821.
[6] Gerth, K. (1985) Anal. Biochem. 144, 432–435.
[7] Fawcett, J.W. and Scott, J.E. (1960) J. Clin. Pathol. 13, 156–159.
[8] Tien, H.T. (1974) Bilayer Lipid Membranes, pp.165–168, Marcel Dekker, New York.
[9] Kashket, E.R. (1981) J. Bacteriol. 146, 377–384.
[10] Kashket, E.R. (1983) FEBS Lett. 154, 343–346.
[11] Hill, S. (1976) J. Gen. Microbiol. 95, 297–312.