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Ammonium and hydroxylamine uptake and accumulation in *Nitrosomonas*

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Starved cells of *Nitrosomonas europaea* and further ammonia oxidizers were able to rapidly accumulate ammonium and hydroxylamine to an internal concentration of about 1 and 0·8 M, respectively. In kinetic studies, the uptake/accumulation rates for ammonium [3·1 mmol (g protein)$^{-1}$ min^{-1}] and hydroxylamine [4·39 mmol (g protein)$^{-1}$ min^{-1}] were determined. The uptake and accumulation process of ammonium and hydroxylamine was not coupled to ammonia or hydroxylamine oxidation and nitrite was not produced. In the presence of uncouplers the ammonium accumulation was completely inhibited, indicating an active, membrane-potential-driven transport mechanism. When the external ammonium or hydroxylamine pool was depleted, the internal ammonium and hydroxylamine was consumed within 12 h or 20 min, respectively. The binding of ammonium/ammonia was correlated with an energized membrane system, and hydroxylamine may bind to the hydroxylamine oxidoredutase.

INTRODUCTION

Ammonia oxidizers like *Nitrosomonas europaea* are a versatile group of micro-organisms found in many natural and engineered ecosystems (Watson *et al.*, 1989; Bock *et al.*, 1995; Schmidt *et al.*, 2001c). Corresponding to this widely spread occurrence their metabolism is flexible; they gain energy using three different pathways, one oxic and two anoxic. The mechanism of aerobic ammonia oxidation was the first pathway discovered (Rees & Nason, 1966; Hooper, 1969a; Hyman & Wood, 1985; Arciero & Hooper, 1993; Hyman & Arp, 1993; Bergmann *et al.*, 1994; Sayavedra-Soto *et al.*, 1994; Dua *et al.*, 1979; Anderson & Hooper, 1983). Later, Abeliovich & Vonshak (1992) and Bock *et al.* (1995) described a heterotrophic and hydrogen-dependent denitrification as an alternative pathway. Evidence for another anoxic mechanism to gain energy was given by Schmidt & Bock (1997) by characterizing the nitrogen dioxide-dependent ammonia oxidation known as the NOx-cycle. This metabolism is obviously the connecting link between anoxic denitrification and oxic nitrification. The nitrogen oxides, NO and NO_2, have been shown to be obligatory intermediates in the oxidation of ammonia, and they have regulatory effects on the metabolism of the nitrifiers (Schmidt & Bock, 1998; Schmidt *et al.*, 2001a–c, 2002; Zart *et al.*, 2000).

The kinetic coefficient for ammonia oxidation was derived from experiments with nitrifying activated sludge, pure cultures, and cell-free extracts of ammonia oxidizers. The K_S value (half saturation constant) for ammonia of ammonia oxidizers like *Nsm. europaea* was found to be fairly constant at about 20 μM between pH 6·5 and 8·5 (Suzuki *et al.*, 1974; Wood, 1986). The current assumption that free ammonia (NH_3) rather than ammonium (NH_4^+) is the substrate for ammonia oxidation in *Nitrosomonas* is based on numerous publications (Suzuki *et al.*, 1974; Drozd, 1976; Hooper & Dispirito, 1985; Painter, 1988; Wiesmann, 1994). Cell membranes are highly permeable for ammonia, in contrast to ammonium, and passive ammonia diffusion over cell membranes to enter the cytoplasm has been discussed as a possible ammonia uptake mechanism (Kleiner, 1981, 1985). Other micro-organisms, like *Corynebacterium glutamicum*, use a membrane-potential-driven transport system for active ammonium uptake (Krämer & Lambert, 1990; Meier-Wagner *et al.*, 2001). Since the ratio between ammonia and ammonium concentration only depends on the pH value, an increase in ammonia in the cytoplasm results in a concomitant increase in ammonium. Therefore, ammonia oxidizers have to tackle several difficulties. The pK_a value (negative logarithm of the dissociation constant for acids) of ammonia is 9·25. As a consequence, at pH values between 7·0 and 8·0 (optimal for most ammonia oxidizers), even at high ammonium concentrations, the ammonia concentration is low. The pH value of the cytoplasm in active

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; HAO, hydroxylamine oxidoredutase.

Nsm. europaea cells is about 6·8–7·2 (Hollocher *et al.*, 1982; Kumar & Nicholas, 1983; C. Look, unpublished data). To establish an ammonia concentration that only meets the K_S value for ammonia oxidation (20 μM, *Nsm. europaea*), the cytoplasmic ammonium concentration should range between 2000 and 5000 μM. If the ammonia uptake over the membrane is limited by diffusion or passive transport, an even higher environmental ammonium concentration would be necessary to establish an internal ammonia concentration of 20 μM. However, the actual ammonium concentration in ecosystems is usually very low (100 μM would result in a maximal cytoplasmic ammonia concentration of 1 μM). It is difficult to envision how ammonia oxidizers can establish a high ammonia oxidation activity that necessitates internal ammonia concentrations in the range of the K_S value (20 μM, *Nsm. europaea*). The ammonium/ammonia concentration was shown to be an important factor for the niche differentiation of ammonia oxidizers (Bollmann *et al.*, 2002), and ammonia oxidizers in niches with low ammonia concentrations will depend on an efficient ammonium/ammonia transport system. Though the genome of *Nsm. europaea* encodes many transporters for inorganic ions, only one ammonium transporter is present, supplementing the passive uptake of ammonium at low pH values (Chain *et al.*, 2003); an active transporter has not yet been described.

The present study shows that starving *Nitrosomonas* cells rapidly take up and accumulate ammonium/ammonia or hydroxylamine without simultaneous nitrite formation. In short-term kinetic analyses, the uptake capacity of ammonium and hydroxylamine of cells and the accumulation capacity of cell-free extracts were determined. ^{15}N-labelling experiments were performed to document that ammonium and hydroxylamine were accumulated, but not immediately oxidized to nitrite. The internal ammonium concentrations of both active and starving cells were determined to provide evidence for an active ammonia/ammonium accumulation.

METHODS

Organisms. Precultures of *Nsm. europaea* (ATCC 19178), *Nitrosomonas eutropha* strain N904 and *Nitrosolobus multiformis* (ATCC 25196) were grown aerobically in 1 l Erlenmeyer flasks containing 400 ml mineral medium (Schmidt & Bock, 1997). The cultures were grown for 1–2 weeks in the dark at 28 °C.

Medium and growth conditions. The mineral medium consisted of the following components (l^{-1} distilled water): NH_4Cl (535 mg), NaCl (585 mg), $CaCl_2.2H_2O$ (147 mg), KCl (74 mg), KH_2PO_4 (54 mg), $MgSO_4.7H_2O$ (49 mg), HEPES (12 g) and 1 ml trace element solution containing 0·02 M HCl (1 l), $FeSO_4.7H_2O$ (973 mg), H_3BO_3 (49 mg), $ZnSO_4.7H_2O$ (43 mg), $(NH_4)_6Mo_7O_{24}.4H_2O$ (37 mg), $MnSO_4.H_2O$ (34 mg), $CuSO_4$ (16 mg). The medium was adjusted to pH 7·2.

Experimental design. All experiments were carried out in Clark-type Oxygen Electrode Units (volume 10 ml) flushed with synthetic air. The oxygen concentration was kept constant at 3 mg l^{-1}. Cells of *Nitrosomonas* and *Nitrosolobus* were harvested from the precultures by centrifugation. The experiments were performed with 'starved cells' or 'active cells'. Before the ammonium/ammonia uptake experiments were started, 'starved cells' were incubated between 20 min and 48 h in the absence of ammonium and hydroxylamine. In contrast, 'active cells' were directly transferred into Clark-type Oxygen Electrode Units to start the experiments (these cells were never incubated without ammonium or hydroxylamine). Experiments were performed at 28 °C with suspensions of 1×10^6–1×10^9 cells ml^{-1}. Medium and biomass samples were transferred into an Eppendorf tube and immediately frozen in liquid nitrogen.

Analytical procedures. Measurements were carried out according to Schmidt & Bock (1997) (ammonium), according to van de Graaf *et al.* (1996) (nitrite, nitrate) and by a modified method according to Verstraete & Alexander (1972) (NH_2OH). The protein concentration was determined according to Bradford (1976). The intracellular pool of ATP was determined by a method according to Strehler & Trotter (1952). Cell numbers were determined by light microscopy with the aid of a Helber chamber. Cell dimensions were evaluated by microscopy (multi-dimensional imaging; Universal Imaging Corporation). Cells were fixed in glutaraldehyde and the cell dimensions were determined with the aid of a distance marker placed on the video screen. Starved and active cells had on average the same volume of 0·2385 fl (0·93 × 0·53 × 0·53 μm).

To measure the ammonium, hydroxylamine and nitrite concentration in the medium, cells were sedimented (5 min at 8000 ***g***) and the supernatant was used for the analytical procedures. To determine the internal (cytoplasmic plus periplasmic) ammonium concentration, the cells were washed (three times) and concentrated in a 0·9 % NaCl solution before homogenization by passing the cell suspension (1×10^{10} cells ml^{-1}) through a French pressure cell at 140 MPa. Samples were taken directly to determine the concentration of free ammonium and hydroxylamine in the cells. The bound ammonium in the cell-free extracts was solubilized by adding an HCl stock solution to a final concentration of 0·1 M, whereas hydroxylamine was solublized by adding detergents (SDS) to a concentration of 10 %. The cell-free extracts were centrifuged (5 min at 8000 ***g***) and the ammonium or hydroxylamine concentrations in the supernatant were measured. The measured concentrations and the cell volume were used to calculate the internal ammonium and hydroxylamine concentrations. The ^{15}N-analysis was performed by isotope ratio MS (Robo-Prep-G and Tracer Mass; Europa Scientific). The ^{15}N-labelled ammonium was analysed after conversion to N_2 with hypobromite (Risgaard-Petersen *et al.*, 1995). The detection limit for all ^{15}N-compounds was 10 nM.

Enzyme purification. Preparation of cell-free extracts and protein purification were performed at 4 °C. *Nitrosomonas* cells were washed three times with 10 mM Tris/HCl (pH 7·5), homogenized by passing the cell suspension (1×10^{10} cells ml^{-1}) through a French pressure cell at 140 MPa, and the crude extract was supplemented with DNase I (Sigma) and centrifuged for 30 min at 48 000 ***g***. The supernatant was loaded onto the HiPrep 16/10 DEAE column (Amersham Pharmacia) and a linear gradient of 0–1 M NaCl was applied at 1 ml min^{-1}. Twenty-five fractions were sampled and their ammonium and hydroxylamine binding capacities were determined. The three fractions with the highest binding capacity were pooled and further purified by anion-exchange chromatography on a UNO Q6 column (Bio-Rad) and eluted by a 0–1 M NaCl gradient. The hydroxylamine-binding fractions were transferred onto an SDS-PAGE gel (Mini-Protean II Cell vertical gel electrophoresis chamber; Bio-Rad) (Schmidt *et al.*, 2001c) and an enzyme assay for hydroxylamine oxidoredutase (HAO) was performed according to Schalk *et al.* (2000).

RESULTS

Ammonium accumulation by ammonia oxidizers

The initial experiments were designed to investigate and compare the ammonium accumulation (uptake) kinetics of both active and starved *Nsm. europaea* cells. Active cells were harvested during the exponential growth phase of a preculture. After the cells were transferred to mineral medium with ammonium, the external and internal ammonium and nitrite concentrations were monitored for 15 min. To perform these kinetic studies with starved cells, the harvested cells were first incubated for 2, 12 or 48 h without ammonium and hydroxylamine. By adding ammonium, the experiment was started. The results are shown in Fig. 1. Several significant differences between active and starved cells were detectable. The internal ammonium concentration of the active ammonia oxidizers was 1 M (Fig. 1b) and the cells started to oxidize ammonium immediately (nitrite was produced within the first 30 s) (Fig. 1a). During starvation the internal ammonium concentration decreased rapidly. When cells had been incubated for 2 h in the absence of ammonium, their internal ammonium concentration decreased from 1 M to about 0·5 M. After 12 h, the ammonium concentration was about 50 μM and after 48 h internal ammonium was depleted (Fig. 1b). Interestingly, the internal ammonium concentration in the starved cells increased dramatically within the first 2 min when ammonium was added (Fig. 1b), but nitrite was not produced (Fig. 1a). The first nitrite was detectable after 5 min and the concentration of about 2 μM was equivalent to only 0·2 % of the ammonium uptake in the first 2 min. When the cells were homogenized to measure the internal ammonium concentration, about 30 % of the ammonium taken up was detectable before the acid treatment and about 100 % after acid treatment. Hence, about 70 % (maximum 0·7 M) of the ammonium was bound and 30 % (maximum 0·3 M) was unbound (free ammonium). The ratio between bound and unbound ammonium (7 : 3) was independent of the total internal ammonium concentration. The nitrite concentration in the cells (internal nitrite concentration) was always identical to the concentration in the medium (cell-free supernatant). Nitrite did not accumulate in the cells. The short lag phase with regard to ammonia oxidation and nitrite production (Fig. 1a) was shown by Hooper (1969b) and most likely this time is required to activate the ammonia oxidation process. As control experiments, cell suspensions with 1×10^6–1×10^9 starved cells ml^{-1} were tested, but the effect of cell density on the internal ammonium concentration was not detectable. The decrease in ammonium concentration in the medium was linearly correlated to the cell density.

To investigate further the energy dependence of the transport mechanism, the influence of protonophors CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and DNP (2,4-dinitrophenol) was investigated. CCCP concentrations between 5 and 200 μM were tested and concentrations from 20 μM upwards completely inhibited ammonium uptake. A DNP concentration of 100 μM (10 μM to 5 mM were tested) reduced the ammonium uptake rate by about 60 % and from 1 mM upwards ammonium uptake was not detectable. The observed instant uptake inhibition after the addition of CCCP or DNP (ammonium uptake was completely inhibited within 1 min), favours a membrane-potential-driven mechanism. The internal ATP pool of the cells remained unaffected for about 5 min at $6{\cdot}9\pm0{\cdot}4$ μmol (g protein)$^{-1}$. The addition of the ATP synthase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD, 40 μM) did not affect ammonium uptake (5 min). Control experiments were performed with *Nsm. eutropha* and *Nsl. multiformis*

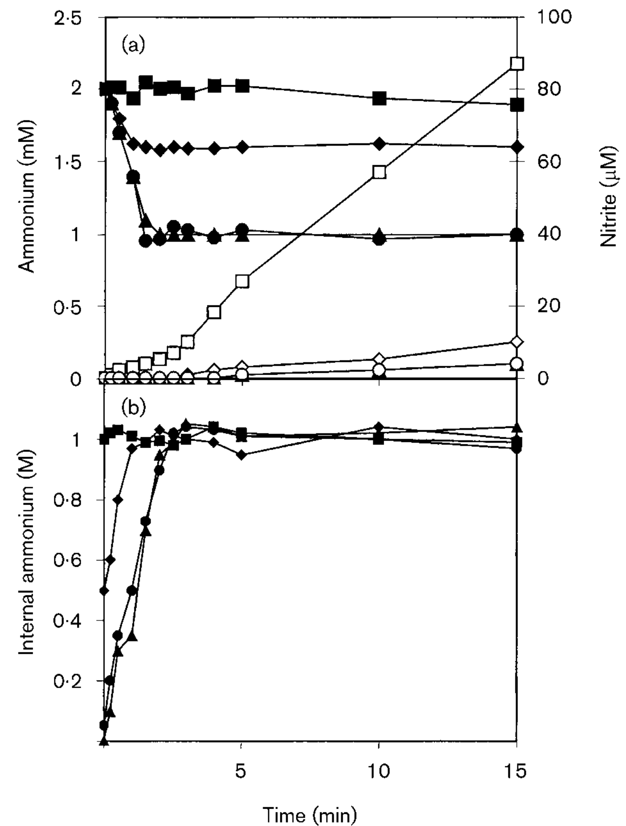

Fig. 1. (a) Ammonium uptake and nitrite production of active and starved (2, 12 or 48 h) *Nsm. europaea* cells (10^9 cells ml^{-1}). Ammonium and nitrite concentrations were determined in the supernatant. The standard deviation for 12 replicated experiments was ±8 %. According to the Mann–Whitney U-Test, the results are highly significant (error rate of 0·005). Black symbols represent the ammonium concentration; white symbols the nitrite concentration in the medium. Squares, active cells; diamonds, starved for 2 h; triangles, starved for 12 h; circles, starved for 48 h. (b) Internal ammonium concentration of active cells and cells starved for 2, 12 or 48 h when exposed to ammonium (time 0). Squares, active cells; diamonds, starved for 2 h; triangles, starved for 12 h; circles, starved for 48 h.

Table 1. Ammonium uptake (accumulation) of *Nsm. eutropha*, *Nsl. multiformis* and cell-free extracts

The standard deviation for ten replicated experiments was ±11 %. According to the Mann–Whitney U-Test, the results are highly significant (error rate of 0·005).

Extract	Internal ammonium concn (M)		Nitrite concn (μM)	
	0 min	2 min	2 min	15 min
*Nsm. eutropha**	0·0	1·2	0·0	11·0
Nsm. eutropha†	1·1	1·05	10·0	125·0
*Nsl. multiformis**	0·0	0·8	0·0	8·0
Nsl. multiformis†	1·0	0·96	6·0	79·0
Cell-free extract of *Nsm. europaea*‡	–	–	0·0	5·0

*Starved cells (incubated for 48 h in ammonium- and hydroxylamine-free medium).
†Active ammonium-oxidizing cells.
‡The protein concentration was equivalent to 10^9 cells ml^{-1}.

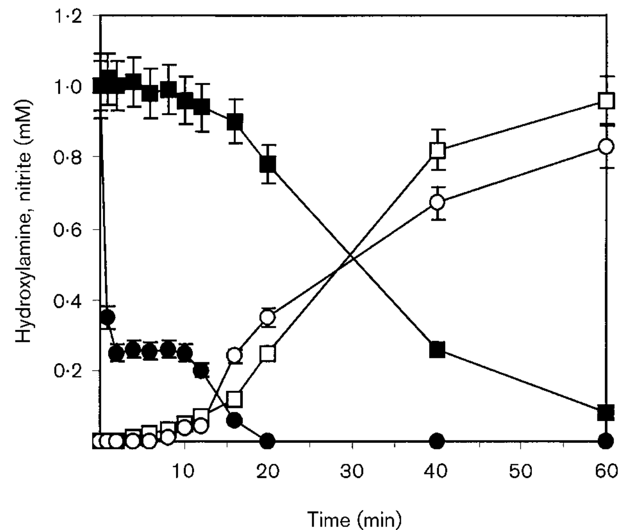

Fig. 2. Hydroxylamine and nitrite concentrations in cell suspensions of active and starved cells of *Nsm. europaea* (10^9 cells ml^{-1}) supplemented with hydroxylamine. The standard deviation for nine replicated experiments was ±11 %. According to the Mann–Whitney U-Test, the results are highly significant (error rate of 0·005). Black symbols represent the hydroxylamine concentrations; white symbols the nitrite concentration. Squares, active cells; circles, starved for 20 min.

(Table 1). Heat-inactivated cells of *Nsm. europaea* (10 min at 50 °C) and further micro-organisms (*Nitrobacter winogradskyi*, *Escherichia coli*, *Paracoccus denitrificans*, *Rhodospirillum rubrum* and *Bacillus subtilis*) were unable to accumulate ammonium or produce nitrite. The ammonium uptake and accumulation abilities *Nsm. eutropha* and *Nsl. multiformis* are similar to those of *Nsm. europaea* (Table 1). During ammonium accumulation, nitrite was not formed. It is interesting to note that cell-free extracts of ammonia oxidizers obviously still have a reduced ability to remove ammonium from the medium. When the cell-free extract was acidified (0·1 M HCl), ammonium was released and 2 mM ammonium was again detectable in the medium.

Hydroxylamine accumulation by ammonia oxidizers

Similarly to the ammonium uptake experiments, hydroxylamine uptake and accumulation activity by the ammonia oxidizers was determined. Active cells did not further accumulate hydroxylamine (internal concentration already at about 0·8 M) and after about 1 min, low concentrations of nitrite were detectable, indicating hydroxylamine oxidation (Fig. 2). During the experiments, hydroxylamine was consumed in stoichiometric amounts relative to the nitrite produced. In contrast, cells kept for only 20 min in a hydroxylamine- and ammonium-free medium (starved cells) showed maximum uptake capacity (Fig. 2). Extended starvation had no further effect. Cell suspensions of 10^9 cells ml^{-1} accumulated hydroxylamine in 1 min to an internal concentration of about 0·8 M. After 2 min 10 μM nitrite was detectable, which equals about 1·25 % of the hydroxylamine uptake in the first minute. Between 2 and 20 min the remaining hydroxylamine in the medium was consumed and equal amounts of nitrite were produced (Fig. 2). When the hydroxylamine in the medium was depleted, the internal hydroxylamine pool was oxidized and the nitrite concentrations in the medium increased (20–60 min). Throughout the experiments, nitrite did not accumulate in the cells and the internal nitrite concentration was similar to the concentration in the medium (±15 %). About 5 % of the internal hydroxylamine was unbound (free hydroxylamine), but 95 % was bound and had to be released by SDS treatment. The ratio of bound to unbound hydroxylamine (19 : 1) was independent of the total internal hydroxylamine concentration. In control experiments with *Ntr. winogradskyi*, *E. coli*, *P. denitrificans*, *R. rubrum*, *B. subtilis*, and in sterile controls the hydroxylamine concentration remained unchanged and nitrite was not produced.

^{15}N-labelling studies

Further uptake experiments were performed with ^{15}N-labelled ammonium and hydroxylamine. Starved and active cells of *Nsm. europaea* were both transferred into mineral medium containing ^{15}N-labelled ammonium or ^{15}N-labelled NH_2OH. Cells were harvested after 0, 2, 5 and 15 min, and the uptake (accumulation) rate of labelled ammonium and hydroxylamine, as well as the nitrite production rate were determined (Table 2). During the first 2 min, starved cells of *Nsm. europaea* rapidly accumulated ammonium or hydroxylamine. Nitrite was not produced in this time interval (the nitrite concentration in the cells and in the cell-free medium remained below the

Table 2. Time-dependent uptake (accumulation) of labelled ammonium and hydroxylamine, and production of nitrite by active and starved *Nsm. europaea* cells (10^9 cells ml^{-1})

The detection limit for all ^{15}N-labelled compounds was 10 nM. The standard deviation for seven replicated experiments was ±9%. According to the Mann–Whitney U-Test, the results are highly significant (error rate of 0·005).

Cell status pre-incubation	Measured ^{15}N-labelled compound	Time-dependent rate [mmol (g protein)$^{-1}$ min^{-1}]		
		0–2 min	2–5 min	5–15 min
Starved	NH_4^+	3·1	0·02	0·08
	NO_2^-	0·0	0·005	0·083
Active	NH_4^+	0·075	0·079	0·077
	NO_2^-	0·069	0·084	0·082
Starved	NH_2OH	4·39	0·3	0·045
	NO_2^-	0·0	0·0	0·061
Active	NH_2OH	0·033	0·034	0·074
	NO_2^-	0·023	0·057	0·092

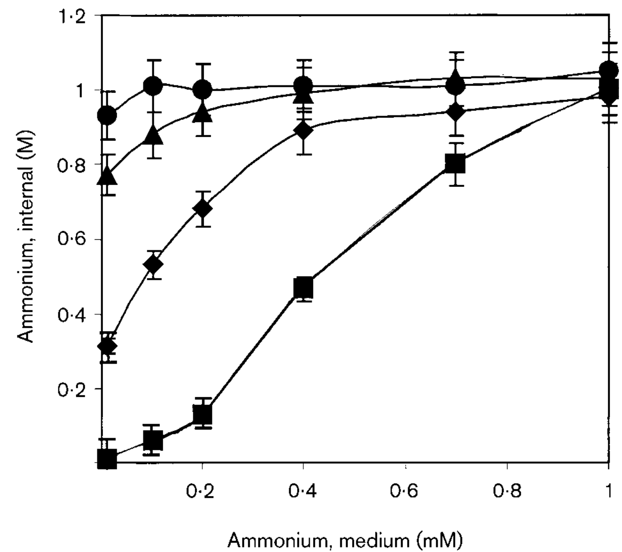

Fig. 3. Internal ammonium concentration of starved *Nsm. europaea* cells that were incubated for 5 min in the presence of 0·01–1 mM ammonium. The cell numbers were adjusted to 10^6 (circles), 10^7 (triangles), 10^8 (diamonds) or 10^9 (squares) cells ml^{-1}. The standard deviation for seven replicated experiments was ±8%. According to the Mann–Whitney U-Test, the results are highly significant (error rate of 0·005).

detection limit of 10 nM). In contrast, the uptake rates (0–2 min) by active cells were low; about 40 times lower for ammonium and about 50 times lower for hydroxylamine compared to the starved cells. Moreover, the uptake of ammonium or hydroxylamine led directly to an almost equivalent production of nitrite.

Ammonium uptake by *Nsm. europaea* cells in media with low ammonium concentrations

On the basis of the findings presented above, it can be speculated that active ammonium uptake and accumulation by ammonia oxidizers is a strategy used to realize high ammonia oxidation activities in ecological niches where ammonium concentrations are low. To evaluate if *Nsm. europaea* is able to accumulate high internal ammonium concentrations at low external concentrations, the internal concentrations after incubation for 5 min at different ammonium concentrations in the medium were determined. To verify a possible effect of the cell density, suspensions with cell numbers between 10^6 and 10^9 cells ml^{-1} were tested (Fig. 3). The results indicate that *Nsm. europaea* cells accumulate ammonium to an internal concentration of about 1 M, independent of the external ammonium concentration. Higher internal concentrations were never observed even if the ammonium concentration in the medium was increased up to 50 mM (not shown).

Ammonium- and hydroxylamine-binding cell components

The ammonium or hydroxylamine taken up by the cells were not immediately oxidized to nitrite, but only small quantities were detectable in the cells (untreated cell-free extracts). However, ammonium could be released from the cell-free extracts by acid treatment (HCl) and hydroxylamine by the addition of detergents (SDS). This indicates that ammonium and hydroxylamine were not converted, but were reversibly bound to a component of the cell. The following experiments aimed at identifying the cell component(s) responsible for the binding of ammonium and hydroxylamine after they were taken up by the cells.

For a first evaluation, crude cell-free extracts of *Nsm. europaea* were separated into extracellular polymer substances (EPS), soluble protein and a membrane fraction. The fractions were supplemented with ammonium or hydroxylamine and the binding capacities were determined (Table 3). The ammonium-binding capacity of all fractions was extremely low. Only in the crude cell-free extracts and in the membrane fraction were small amounts of ammonium bound. The hydroxylamine-binding capacity was high in both the crude cell-free extract and in the soluble protein fraction. To elucidate which proteins were responsible for hydroxylamine binding, the soluble protein fraction was further separated. After each separation step the fractions with the highest hydroxylamine-binding capacity were pooled. After purification, the hydroxylamine-binding protein was identified as HAO by SDS-PAGE and an enzyme assay. On SDS gels, proteins of 125 and 65 kDa were detectable, which resemble the masses of the HAO (125–140 kDa) and its subunits (63 kDa) (Masson *et al.*, 1990; Nejidat *et al.*, 1997; Arciero & Hooper, 1993). In the enzyme assay, a hydroxylamine oxidation activity of

Table 3. Binding capacities of different cell fractions of *Nsm. europaea*

The binding capacity was measured in kinetic studies based on the removal of free ammonium or hydroxylamine from the medium. Twelve replicated experiments were performed.

Cell component	Binding capacity for:	
	Ammonium [μmol (g dry wt)$^{-1}$]	Hydroxylamine [μmol (g dry wt)$^{-1}$]
Crude cell-free extract	17 ± 6·3	161 ± 34·2
EPS	0 ± 0	5 ± 2·2
Soluble protein	3 ± 0·9	278 ± 47·8
Membrane	23 ± 5·7	16 ± 2·9
Purified HAO	7 ± 1·5	468 ± 50·1†
Purified HAO (heated)*	6 ± 2·1	4 ± 1·3

*HAO was incubated for 3 min at 90 °C before supplementation with hydroxylamine.

†Molar ratio between hydroxylamine and HAO is about 58·5 (HAO, 125 kDa).

58 μmol (mg protein)$^{-1}$ min^{-1} was measured. The binding of hydroxylamine to HAO was a reversible process. Following addition of SDS (10 %), the bound hydroxylamine was released from the protein and was detectable in the medium. Hence, hydroxylamine was not covalently bound to HAO. Heat-treated HAO (3 min at 90 °C) was unable to bind hydroxylamine, indicating that a native protein is necessary for the binding process.

DISCUSSION

Ammonium and hydroxylamine uptake by ammonia oxidizers has been thought to be a passive diffusion process across the membrane (Kleiner 1981, 1985). The first indications of an ammonium transporter which might supplement the passive uptake at low pH were provided by Chain *et al.* (2003). Our current study provides evidence for an internal accumulation of ammonium and hydroxylamine by a maximal factor of 10^5.

The major results of this study are as follows. First, all ammonia oxidizers tested (*Nsm. europaea*, *Nsm. eutropha*, *Nsl. multiformis*) accumulated ammonium to a final internal concentration of about 1 M (about 6×10^8 molecules per cell). Second, this internal ammonium concentration remained constant during ammonia oxidation, but decreased rapidly when the external ammonium was consumed. Third, starved cells accumulated 1 M ammonium before the first catabolic products (nitrite) were detected, and the specific ammonium uptake rate was about 40 times higher than the highest specific ammonia oxidation activity observed. Fourth, inactive cells and cell components were unable to bind significant amounts of ammonium/ammonia. Fifth, the results provide evidence for a membrane-potential-driven ammonium uptake mechanism.

According to the results of ^{15}N-labelling studies the processes of ammonium uptake/accumulation and catabolic ammonia oxidation forming nitrite have to be distinguished. Starved cells accumulate ^{15}N-labelled ammonium and increase their internal ammonium concentration to about 1 M. Throughout this time period ammonia is not oxidized and ^{15}N-labelled nitrite is not formed. Cells might need several minutes to recover their ammonia oxidation activity (Hooper, 1969b) or they have to establish a sufficient internal ammonia concentration before oxidation starts. In contrast, in experiments with active cells (internal ammonium concentration already 1 M) ammonia was oxidized immediately and stochiometric amounts of nitrite were produced.

Ammonia oxidizers have to tackle substrate limitations in many environments. At an ammonium concentration of 100 μM, the ammonia concentration is about 0·8 μM at pH 7. Passive transport (diffusion) over the membrane would be a slow process and even an internal ammonia concentration of about 0·8 μM would be significantly below the K_S value of ammonia monooxygenase (20 μM, *Nsm. europaea*), resulting in a low specific ammonia oxidation activity. This ammonia limitation can be compensated by internal ammonium accumulation. At an internal ammonium concentration of 1 M, as measured in this study, an ammonia concentration of about 80 mM would be available, which is 500 times higher than the K_S value of the ammonia-oxidizing system. Ammonium accumulation might be a strategy adopted by ammonia oxidizers in environmental niches with low ammonium concentrations to maintain high ammonia oxidation activities. This accumulation strategy would also protect the ammonia-oxidizing enzyme system from an evolutionary pressure to increase its affinity for ammonia, but instead this pressure is placed on the effectiveness of the ammonium/ammonia uptake (transport) system, since ammonia oxidizers have to compete with other organisms (heterotrophic micro-organisms, fungi, algae, plants) for ammonium (Laanbroek & Gerards, 1993; Bodelier & Frenzel, 1999). Although ammonia oxidizers are able to establish high internal ammonium concentrations, this pool has obviously no function as an ammonium (substrate) stock. When the ammonium in the medium was depleted, the internal ammonium concentration decreased rapidly. Possibly, the internal ammonium pool is used to prepare the ammonia oxidizers for starvation, for example by synthesizing compatible solutes (Prosser, 1989).

The results presented indicate that ammonium uptake (transport) is a membrane-potential-driven mechanism [proton motive force (pmf)-dependent]. First, the instant uptake inhibition after addition of CCCP or DNP favours pmf-driven transport (Krämer & Lambert, 1990; Meier-Wagner *et al.*, 2001). Second, the ATP pool of the cells and

subsequently primary ATP-driven transport systems remained unaffected for several minutes. ATP would have been accessible if necessary for ammonium transport. Third, supplementation of ATP synthase inhibitors had no effect on the uptake rates for several minutes. Also, hydroxylamine uptake is most likely to be based on an active transport mechanism. At 40 mM the internal free hydroxylamine concentration was at least 40 times higher than the external hydroxylamine concentration in the medium. Together with the high uptake rate, these results provide evidence for active hydroxylamine transport.

Experiments investigating the binding capacity of ammonium/ammonia (*Nsm. europaea*) showed only low binding capacity in the membrane fraction and the crude cell-free extract. It seems possible that the binding of ammonium is dependent on an energized membrane and that the positively charged ammonium molecules are electrostatically bound to the negatively charged side of cytoplasmic membrane. Hydroxylamine was shown to be bound to HAO (Table 3) and the addition of SDS displaced it. The process of hydroxylamine binding was dependent on a native protein. A model of the surface charge of native HAO shows extensive negatively or positively charged areas (Igarashi *et al.*, 1997). This allows an electrostatic interaction between the enzyme and the (partially) charged hydroxylamine molecule, leading to its immobilization.

The results presented above strongly indicate an active ammonium transport system in ammonia oxidizers. Analysis of the *Nsm. europaea* genome provides evidence for an ammonium transporter that might support ammonium uptake at low pH values (Chain *et al.*, 2003). Database analysis of the protein (ORF2457) revealed a high level of homology to a family of active ammonium transporters in, for example, *E. coli*, *B. subtilis* and *Saccharomyces cerevisiae*, containing domains for an Na^+/H^+ antiporter and cation transport. Furthermore, the genome encodes 13 putative active transporters (ABC), including one nitrate transport system (Chain *et al.*, 2003). It can be speculated that a modified ABC-type transporter might be responsible for the active ammonium uptake by ammonia oxidizers.

REFERENCES

Abeliovich, A. & Vonshak, A. (1992). Anaerobic metabolism of *Nitrosomonas europaea*. *Arch Microbiol* **158**, 267–270.

Anderson, K. K. & Hooper, A. B. (1983). O_2 and H_2O are each the source of one O in NO_2^- produced from NH_3 by *Nitrosomonas*; ^{15}N-NMR evidence. *FEBS Lett* **164**, 236–240.

Arciero, D. M. & Hooper, A. B. (1993). Hydroxylamine oxidoreductase from *Nitrosomonas europaea* is a multimer of an octaheme subunit. *J Biol Chem* **268**, 14645–14654.

Bergmann, D. J., Arciero, D. A. & Hooper, A. B. (1994). Organization of the *hao* gene cluster of *Nitrosomonas europaea*: genes for two tetraheme *c* cytochromes. *J Bacteriol* **176**, 3148–3153.

Bock, E., Schmidt, I., Stüven, R. & Zart, D. (1995). Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. *Arch Microbiol* **163**, 16–20.

Bodelier, P. L. & Frenzel, P. (1999). Contribution of methanotrophic and nitrifying bacteria to CH_4 and NH_4^+ oxidation in the rhizosphere of rice plants as determined by new methods of discrimination. *Appl Environ Microbiol* **65**, 1826–1833.

Bollmann, A., Bar-Gilissen, M. J. & Laanbroek, H. J. (2002). Growth at low ammonium concentrations and starvation response as potential factors involved in niche differentiation among ammonia-oxidizing bacteria. *Appl Environ Microbiol* **68**, 4751–4757.

Bradford, M. (1976). Rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**, 248–254.

Chain, P., Lamerdin, J., Larimer, F. & 12 other authors (2003). Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J Bacteriol* **185**, 2759–2773.

Drozd, J. W. (1976). Energy coupling and respiration in *Nitrosomonas europaea*. *Arch Microbiol* **110**, 257–262.

Dua, R. D., Bhandari, B. & Nicholas, D. J. D. (1979). Stable isotope studies on the oxidation of ammonia to hydroxylamine by *Nitrosomonas europaea*. *FEBS Lett* **106**, 401–404.

Hollocher, T. C., Kumar, S. & Nicholas, D. J. D. (1982). Respiration-dependent proton translocation in *Nitrosomonas europaea* and its apparent absence in *Nitrobacter agilis* during inorganic oxidations. *J Bacteriol* **149**, 1013–1020.

Hooper, A. B. (1969a). Biochemical basis of obligate autotrophy in *Nitrosomonas europaea*. *J Bacteriol* **97**, 776–779.

Hooper, A. B. (1969b). Lag phase of ammonia oxidation by resting cells of *Nitrosomonas europaea*. *J Bacteriol* **97**, 968–969.

Hooper, A. B. & DiSpirito, A. A. (1985). In bacteria which grow on simple reductants generation of a proton gradient involves extracytoplasmic oxidation of substrate. *Microbiol Rev* **49**, 140–157.

Hyman, M. R. & Arp, D. J. (1993). An electrophoretic study of the thermal-dependent and reductant-dependent aggregation of the 28 kDa component of ammonia monooxygenase from *Nitrosomonas europaea*. *Electrophoresis* **14**, 619–627.

Hyman, M. R. & Wood, P. M. (1985). Suicidal inactivation and labeling of ammonia monooxygenase by acetylene. *Biochem J* **227**, 719–725.

Igarashi, N., Moriyama, H., Fujiwara, T., Fukumori, Y. & Tanaka, N. (1997). The 2·8 Å structure of hydroxylamine oxidoreductase from nitrifying chemoautotrophic bacterium *Nitrosomonas europaea*. *Nat Struct Biol* **4**, 276–284.

Kleiner, D. (1981). The transport of NH_3 and NH_4^+ across biological membranes. *Biochim Biophys Acta* **639**, 41–52.

Kleiner, D. (1985). Bacterial ammonia transport. *FEMS Microbiol Rev* **32**, 87–100.

Krämer, R. & Lambert, C. (1990). Uptake of glutamate in *Corynebacterium glutamicum*. 2. Evidence for a primary active transport system. *Eur J Biochem* **194**, 937–944.

Kumar, S. & Nicholas, D. J. D. (1983). Proton electrochemical gradients in washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis*. *J Bacteriol* **154**, 65–71.

Laanbroek, H. J. & Gerards, S. (1993). Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradskyi* grown in continuous mixed cultures. *Arch Microbiol* **159**, 453–459.

Masson, P., Arciero, D. M., Hooper, A. B. & Balny, C. (1990). Electrophoresis at elevated hydrostatic pressure of the multiheme hydroxylamine oxidoreductase. *Electrophoresis* **11**, 128–133.

Meier-Wagner, J., Nolden, L., Jakoby, M., Siewe, R., Krämer, R. & Burkovski, A. (2001). Multiplicity of ammonium uptake systems in *Corynebacterium glutamicum*: role of Amt and AmtB. *Microbiology* **147**, 135–143.

Nejidat, A., Shmuely, H. & Abeliovich, A. (1997). Effect of ammonia starvation on hydroxylamine oxidoreductase activity of *Nitrosomonas europaea*. *J Biochem* **121**, 957–960.

Painter, H. A. (1988). Nitrification in the treatment of sewage and waste-waters. In *Nitrification*, pp. 185–211. Edited by J. I. Prosser. Oxford: IRL Press.

Prosser, J. I. (1989). Autotrophic nitrification in bacteria. *Adv Microb Physiol* **30**, 125–181.

Rees, M. & Nason, A. (1966). Incorporation of atmospheric oxygen into nitrite formed during ammonia oxidation by *Nitrosomonas europaea*. *Biochim Biophys Acta* **113**, 398–401.

Risgaard-Petersen, N., Rysgaard, S. & Revsbech, N. P. (1995). A combined microdiffusion–hypobromite oxidation method for determination of ^{15}N isotope in NH_4^+. *Soil Sci Soc Am J* **59**, 1077–1080.

Sayavedra-Soto, L. A., Hommes, N. G. & Arp, D. J. (1994). Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea*. *J Bacteriol* **176**, 504–510.

Schalk, J., Devries, S., Kuenen, J. G. & Jetten, M. S. M. (2000). A novel hydroxylamine oxidoreductase involved in the Anammox process. *Biochemistry* **39**, 5405–5412.

Schmidt, I. & Bock, E. (1997). Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. *Arch Microbiol* **167**, 106–111.

Schmidt, I. & Bock, E. (1998). Anaerobic ammonia oxidation by cell-free extracts of *Nitrosomonas eutropha*. *Antonie van Leeuwenhoek* **73**, 271–278.

Schmidt, I., Zart, D. & Bock, E. (2001a). Gaseous NO_2 as a regulator for ammonia oxidation of *Nitrosomonas eutropha*. *Antonie van Leeuwenhoek* **79**, 311–318.

Schmidt, I., Zart, D. & Bock, E. (2001b). Effects of gaseous NO_2 on cells of *Nitrosomonas eutropha* previously incapable of using ammonia as an energy source. *Antonie van Leeuwenhoek* **79**, 39–47.

Schmidt, I., Bock, E. & Jetten, M. S. M. (2001c). Ammonia oxidation by *Nitrosomonas eutropha* with NO_2 as oxidant is not inhibited by acetylene. *Microbiology* **147**, 2247–2253.

Schmidt, I., Sliekers, O., Schmid, M., Cirpus, I., Strous, M., Bock, E., Kuenen, J. G. & Jetten, M. S. M. (2002). Aerobic and anaerobic ammonia oxidizing bacteria – competitors or natural partners? *FEMS Microbiol Ecol* **39**, 175–181.

Strehler, B. L. J. & Trotter, J. B. (1952). Firefly luminescence in the study of energy transfer mechanism. I. Substrate and enzyme determination. *Arch Biochim Biophys* **40**, 28–41.

Suzuki, I., Dular, U. & Kwok, S.-C. (1974). Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. *J Bacteriol* **120**, 556–558.

Van de Graaf, A. A., de Bruijn, P., Robertson, L. A. & Kuenen, J. G. (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology* **142**, 2187–2196.

Verstraete, W. & Alexander, M. (1972). Heterotrophic nitrification by *Arthrobacter* sp. *J Bacteriol* **110**, 955–961.

Watson, S. W., Bock, E., Harms, H., Koops, H.-P. & Hooper, A. B. (1989). Genera of ammonia-oxidizing bacteria. In *Bergey's Manual of Systematic Bacteriology*, pp. 1822–1834. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.

Wiesmann, U. (1994). Biological nitrogen removal from wastewater. *Adv Biochem Eng Biotechnol* **51**, 113–154.

Wood, P. M. (1986). Nitrification as a bacterial energy source. In *Nitrification*, pp. 39–62. Edited by J. I. Prosser. Oxford: IRL Press.

Zart, D., Schmidt, I. & Bock, E. (2000). Significance of gaseous NO for ammonia oxidation by *Nitrosomonas eutropha*. *Antonie van Leeuwenhoek* **77**, 49–55.