AMMONIA OXIDATION BY CELL-FREE EXTRACTS OF NITROSOCYSTIS OCEANUS[1]

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SUMMARY: Cell-free extracts prepared by rupturing cells of Nitrosocystis oceanus in the presence of seawater, Tris, ATP, magnesium, and phosphate oxidize ammonia to nitrite.

To date ammonia oxidase activity in cell-free extracts of nitrifying bacteria has not been shown. In the present study we wish to report the cell-free oxidation of ammonia by N. oceanus, a marine ammonia oxidizing bacterium, and to present further evidence suggesting that hydroxylamine is an intermediate in this reaction.

The members of the genera Nitrosomonas and Nitrosocystis are obligate chemolithotrophs which fulfill their major energy and carbon needs by the oxidation of ammonia and the fixation of carbon dioxide. Since the oxidation of ammonia to nitrite involves the removal of six electrons, it has been proposed that this oxidation occurs through a series of intermediates. However, previous investigators have been unable to demonstrate ammonia oxidation in cell-free extracts, so the exact nature of these intermediates has not been defined. As early as 1926, Kluyver and Donker (1) suggested that hydroxylamine was an intermediate in this reaction. Further credence was given to this theory when Hofman and Lees (2, 3), using the inhibitor hydrazine, demonstrated the accumulation of hydroxylamine when whole cells of Nitrosomonas europaea oxidized

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ammonia to nitrite. Evidence substantiating this theory also came from the work of Nicholas and Jones (4) who showed that cell-free extracts of N. europaea oxidized hydroxylamine. Since these initial studies numerous investigators have verified the cell-free oxidation of hydroxylamine by nitrifying bacteria, but have not demonstrated that ammonia could be oxidized in these extracts.

METHODS AND MATERIALS: A pure culture of N. oceanus was maintained in continuous culture in a 35 liter pH-stat fermentor (5). The cells were harvested in the early phase of logarithmic growth by means of a refrigerated (5°C) continuous flow centrifuge (Sorvall). After harvesting, the cells were washed 5 times with filtered seawater. Finally, the cells were suspended in their rupturing media at a weight/volume ratio of 1:20. Some were ruptured in distilled water which was 20 mM in Tris-Cl, pH 7.6 (distilled water-Tris) or seawater which was 20 mM in Tris-Cl, pH 7.6 (seawater-Tris) by means of a French Pressure Cell (American Instrument Co., Inc.) at 20,000 psi; the remainder was ruptured in the presence of magnesium (10 mM), ATP (1 mM), and phosphate (10 mM) in both distilled water-Tris and seawater-Tris. The extracts were spun for 10 minutes at 1,500 x g and again at 3,000 x g to insure the removal of any whole cells. The resulting supernatants were spun at 39,000 x g for 15 minutes and again at 144,000 x g for 60 minutes. All pellets were suspended in their original rupturing media.

The ammonia oxidase activity of all fractions was determined by measuring the production of nitrite from ammonia (6). Each fraction was incubated in distilled water-Tris or seawater-Tris with 5 mM ammonia. Comparable assays were run with 50 μM hydroxylamine as substrate. Proteins were determined by the methods of Lowry et al. (7) and Gornall et al. (8).

All fractions were negatively stained with 1% phosphotungstic

acid pH 7.0 and examined in a Philips 300 electron microscope at an accelerating voltage of 60 kv.

TABLE I

Ammonia and hydroxylamine oxidase activities compared in various breakage media

Substrate	I Distilled water-Tris	II Distilled water-Tris ATP, Mg^{++}, $PO^{=}_4$	III Seawater-Tris	IV Seawater-Tris ATP, Mg^{++}, $PO^{=}_4$
Ammonia 5 mM	0.0	0.7	5.0	16.0
Hydroxylamine 50 μM	0.3	1.9	15.5	20.0

Values are expressed in millimicromoles nitrite per minute per milligram protein. All systems were assayed with the 39,000 x g pellet and were buffered with 20 mM Tris-Cl, pH 7.5. The final concentrations of the additives were ATP (1 mM), Mg^{++} (10 mM), and $PO^{=}_4$ (10 mM).

TABLE II

Nitrite production from ammonia and hydroxylamine in cell-free extracts with and without additives

Substrate	Whole Cells	French Press Initial Fractions	39,000 x g Supernatant	39,000 x g pellet	144,000 x g Supernatant	144,000 x g pellet
Ammonia 5 mM						
a. no additives	230	1.2	0.0	5.0	0.0	0.0
b. plus additives	230	22.0	0.3	16.0	0.0	0.0
Hydroxylamine 50 μM						
a. no additives	18*	10.0	3.1	15.5	0.1	14.0
b. plus additives	17*	11.0	3.0	20.1	0.2	14.0

Values are expressed in millimicromoles nitrite per minute per milligram protein. All fractions were in seawater-Tris. Concentrations of additives were ATP (1 mM), Mg^{++} (10 mM), and $PO^{=}_4$ (10 mM).

*When the optimal hydroxylamine concentration of 1.0 mM was used in the whole cell fractions, these values were 66 and 70 respectively.

RESULTS: Cells of N. oceanus grown in continuous culture and ruptured in seawater, Tris, Pi, ATP and magnesium oxidized ammonia to nitrite 100% of the time. Cells ruptured in seawater-Tris without ATP, magnesium and Pi oxidized ammonia about 25% of the time whereas cells crushed in distilled water-Tris showed minimal activity (Table I). When ATP, Pi, and magnesium were added to either seawater-Tris or distilled water-Tris, the oxidation rate increased. However the rates were considerably greater in the seawater system where they approximated as much as 20% of the whole cell activity.

To demonstrate cell-free ammonia oxidation, cells had to be grown under controlled cultural conditions. If the nitrite concentration in the growth medium exceeded 12 mM, ammonia oxidation could rarely be demonstrated. It was also essential that the cells be grown at pH 7.5-7.8 and harvested in the exponential growth phase. Since nitrite is inhibitory, harvested cells were washed until the nitrite level was below 5μM.

Cell-free extracts were fractionated stepwise by differential centrifugation (Table II). Ammonia oxidase activity was found to be concentrated in the 39,000 x g pellet (Table II). When this fraction was viewed either with a phase-contrast or electron microscope, no whole cells or partially lysed cells were evident. Electron microscopy showed that this pellet (Fig. 1) contained vesicles covered with 80-100 Å doughnut shaped particles.

Since the major fraction of the ammonia oxidase was in the 39,000 x g pellet, it was assumed that most or all of these enzymes were particulate and likely membrane bound. The fact that the 39,000 x g pellet was comprised primarily of membranes seemed to substantiate this hypothesis.

In contrast to the ammonia oxidase activity the hydroxylamine oxidase activity was similar in both the 39,000 and 144,000 x g pellets (Table II). The hydroxylamine oxidase activity also differed from the

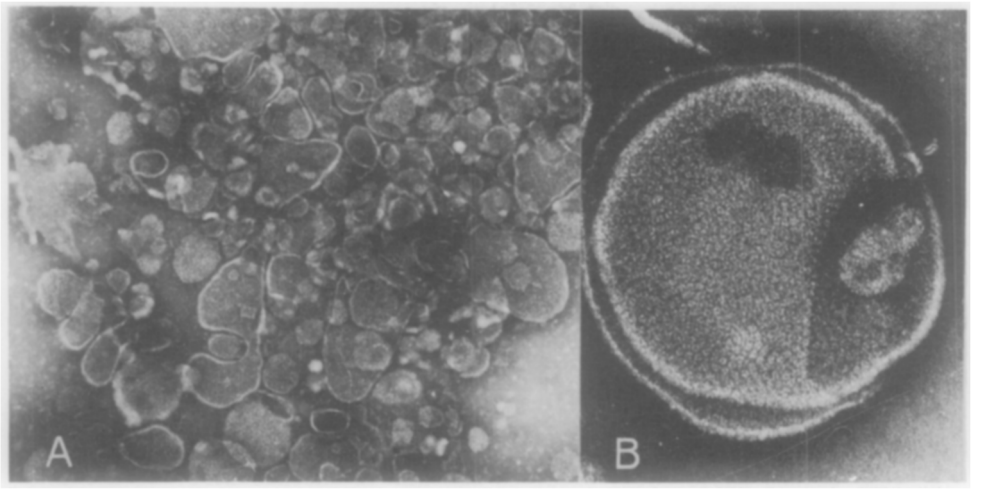

Figure 1 A. Electron micrograph of the 39,000 g pellet, X 20,700.

B. Membrane fragment from 39,000 g pellet showing 80-100 Å particles, X 126,800.

ammonia oxidase activity in that additives were not needed for maximum activity (Table II). These facts suggest that the additives were needed to stabilize or stimulate the oxidation of ammonia.

In cell-free extracts the optimal ammonia and hydroxylamine concentrations were 5 mM and 50 μM respectively; higher hydroxylamine concentrations were toxic. In whole cells the optimal concentration of hydroxylamine was 1.0 mM, and the rate of substrate oxidation was three to ten times greater than that found in cell-free extracts.

Ammonia and hydroxylamine were oxidized at approximately the same rate in cell-free extracts. This observation is consistent with previous suggestions (2, 3) that hydroxylamine may be a metabolic intermediate between ammonia and nitrite.

While our results indicated that magnesium and ATP were needed for the oxidation of ammonia to hydroxylamine, experimental information concerning the role played by these additives was lacking. Occasionally when cells were crushed in the presence of magnesium (20-40 mM), the 80-100 Å particles covering the membranes were arranged in a recti-

linear array. In the absence of magnesium the particles were not aligned in a symmetrical or regular manner. This suggested that magnesium may have been needed to maintain an ordered spatial arrangement of the particles on the membranes.

In addition to magnesium, monovalent cations were also required for the oxidation of ammonia. Since N. oceanus is a marine organism, it normally grows in a medium with a sodium concentration of approximately 0.45M. While this organism cannot be grown in the absence of sodium, washed whole cells oxidized ammonia if another monovalent cation was substituted for sodium. In the absence of monovalent cations no ammonia was oxidized. In cell-free extracts no specific attempt was made to substitute other monovalent cations for sodium.

Ammonia oxidation in cell-free extracts can be demonstrated occasionally in the absence of ATP. Probably its requirement is contingent upon the physiological state of the cells. It has been reported that the oxidation of ammonia to hydroxylamine is an energy requiring action (9). Whether ATP is supplying the energy supposedly needed to drive this reaction cannot be determined from our present experimental data.

It is interesting to note that both magnesium and ATP along with succinate are needed for the preparation of phosphorylating submitochondrial particles (10). In both cases magnesium and ATP may be required to maintain the membranes in a biochemically and structurally active state.

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