NITROGEN ASSIMILATION AND ENERGY CONSERVATION
IN NITROSOMONAS EUROPaea AND NITROBACTER AGIJS

by

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SUMMARY

1. This thesis embodies results of an investigation on some aspects of nitrogen assimilation and energy conservation in nitrifying bacteria, *Nitrosomonas europaea* and *Nitrobacter agilis*.

2. Electrode techniques have been developed to measure NO$_3^-$ production and O$_2$ uptake simultaneously and continuously during NO$_2^-$ utilization by *Nitrobacter agilis*. The stoichiometry of NO$_2^-$ oxidation by washed cells was 1NO$_2^-$ : 0.5O$_2$ : 0.75NO$_3^-$ compared to 1NO$_2^- : 0.50_2 : 1NO_3^-$ for spheroplasts and membrane vesicles of *Nitrobacter agilis*. The effects of several metabolic inhibitors on NO$_2^-$ and O$_2$ utilization and NO$_3^-$ production were investigated. Nitrite oxidation was found to be particularly sensitive to the inhibitors of cytochrome oxidase (eg. azide), ATPase (eg. DCCD), -SH group (eg. pCMB) and uncouplers of oxidative phosphorylation (eg. CCCP).

3. A Mg$^{2+}$ dependent ATPase was detected in membrane vesicles of *Nitrobacter agilis* which was inhibited by classical ATPase inhibitors (eg. DCCD) but was unaffected by uncouplers (eg. 2,4-DNP, CCCP). The ATPase activity of membrane vesicles was progressively lost by delipidation of membranes by phospholipase A$_2$.

4. The growth yields of *Nitrobacter agilis* were increased about 2 fold by growing the bacterium in a NO$_2^-$ medium supplemented with 2mM-NH$_4$Cl. Higher concentrations of NH$_4$Cl however competitively inhibited NO$_2^-$ oxidation and the growth of the bacterium.

5. Washed cells of *Nitrobacter agilis* readily incorporated $^{15}$NH$_4^+$, $^{15}$NH$_2$OH, $^{15}$NO$_2^-$ and $^{15}$NO$_3^-$ respectively (in decreasing order) into cell nitrogen inhibitors of glutamine synthetase (L-methionine DL-sulphoxide) and glutamate synthase (serine) did not affect the incorporation of either $^{15}$NH$_4^+$ or $^{15}$NO$_2^-$ into cell nitrogen, indicating that glutamate dehydrogenase pathway is the main route for the assimilation of NH$_4^+$ in *Nitrobacter agilis*.

6. Glutamine synthetase was purified 400 fold from *Nitrobacter agilis* and its properties and regulation studied. The enzyme (molecular weight 200,000) which contained 12 subunits of 58,000 each was regulated by feed back inhibition involving amino acids and nucleotides, substrate
inhibition by NH₄⁺ as well as by an adenylylation/deadenylylation mechanism. An isoactivity pH of 7.4 was recorded for the purified enzyme.

7. Glutamine synthetase from Nitrosomonas europaea was purified 710 fold. In crude extracts, the Mg²⁺ effect on the γ-glutamyl transferase activity was related to NH₄⁺ concentration in the growth medium. This enzyme activity was stimulated two fold by Mg²⁺ in crude extracts from cells of culture from which NH₄⁺ had been depleted. Unlike the Nitrobacter enzyme, the γ-glutamyl transferase activity of either crude extracts or the purified enzyme from Nitrosomas europaea was unaffected by snake venom phosphodiesterase treatment.

8. Two isoforms of glutamate dehydrogenase specific for NAD⁺ and NADP⁺ respectively were detected in the cytosol fraction of Nitrobacter agilis. The NAD⁺ enzyme functioned in both directions i.e. ammination and deamination whereas the NADP⁺ enzyme was primarily for the amination of α-ketoglutarate to glutamate. The NADP⁺ enzyme was purified 52 fold (free of NAD⁺ enzyme) by affinity chromatography on 2′-5′ADP Sepharose-4B and some of its properties studied. Substrate activation of the NADP⁺ enzyme was observed with NH₄⁺ and NADPH. A comparison was made of the properties of the purified NADP⁺ enzyme from Nitrobacter agilis with that from Nitrosomonas europaea.

9. Oxygen pulse experiments were carried out with washed cells of Nitrosonomas europaea and Nitrobacter agilis and with spheroplasts and everted membrane vesicles prepared from Nitrobacter agilis. In addition to thiocyanate, the salting-in anions perchlorate and trichloroacetate and lipophilic cation triphenyl methyl phosphonium (TPMP⁺) proved to be permeant and effective in allowing respiration-dependent proton translocation in cells of Nitrosomonas europaea. The observed +H⁺/O ratio for NH₄⁺ oxidation by Nitrosomonas europaea was 3.4 and that for both NH₄OH and N₂H₅⁺ oxidation was 4.4. These values when corrected for the production of stoichiometric protons and for the fact that the first step in NH₄⁺ oxidation is mediated by a mono-oxygenase, gave effective +H⁺/O ratios of about 4 for these three substrates. No convincing evidence was obtained for the operation of a respiratory proton pump in Nitrobacter agilis during NO₂⁻ oxidation.

10. The components of proton-motive force (Δp), namely membrane potential (Δψ) and transmembrane pH gradient (ΔpH) were determined in washed cells of Nitrosomonas europaea and Nitrobacter agilis. In these bacteria, both Δψ and ΔpH were dependent on external pH (pHₑ). Thus at pHₑ 8 cells of
Nitrosomonas europaea and *Nitrobacter agilis* had Δψ of 175mV and 125mV (inside negative) respectively as determined by the distribution of \(^3H\) tetraphenyl phosphonium cation (TPP\(^+\)). Intracellular pH was determined by the distribution of \(^{14}C\) benzoic and \(^{14}C\) acetyl salicylic acids and \(^{14}C\) methylene. At pH 7 for *Nitrosomonas europaea* and 7.3 for *Nitrobacter agilis* there was no detectable ΔpH so that only Δψ contributed to Δp. Intracellular pH (pHi) in cells of *Nitrosomonas europaea* varied from 6.2 at pH 6 to 7.8 at pH 8.5. In *Nitrobacter agilis* however pH was relatively constant (7.3 to 7.8) over the pH range of 6 to 8.5. The components of Δp (Δψ and ΔpH) remained constant at various stages of growth of *Nitrosomonas europaea* so that the metabolic state of the cells did not affect Δp. Such an experiment was not possible with *Nitrobacter agilis* because of low cell yields.

11. Spheroplasts of *Nitrosomonas europaea* synthesized ATP in response to an artificially created Δp. This ATP synthesis was inhibited by DCCD indicating that it was mediated by an ATPase.

12. Cation (Na\(^+\), K\(^+\) and NH\(_2\)\(^+\)) transport systems in *Nitrosomonas europaea* and *Nitrobacter agilis* were investigated. In K\(^+\) depleted cells it was shown that K\(^+\) is transported by an electrogenic mechanism in both bacteria and its uptake resulted in partial conversion of Δψ into ΔpH. NH\(_2\)\(^+\) was transported essentially as a neutral species (NH\(_4\)) by a simple diffusion mechanism. Experiments with \(^22Na\) loaded cells indicated that antiporters for Na\(^+\)/H\(^+\), Na\(^+\)/K\(^+\) and K\(^+\)/H\(^+\) were present in both bacteria. At least one of these antiporters (Na\(^+\)/K\(^+\)) required an electrochemical gradient for its operation.

13. Using stable isotopes of \(_{15}N\) (\(^{15}NO\(_2\)\) and \(^{15}NO\(_3\)) and \(^{18}O\) (H\(_2\)\(^{18}O\), \(^{18}O\(_2\) and P\(^{18}O\(_2\)) it was shown by GC/MS and \(^{15}N\)-NMR techniques that the third 'O' in \(^{15}NO\(_3\) produced by \(^{15}NO\(_2\) oxidation by cells of *Nitrobacter agilis* originated from \(^{18}O\(_2\) and not from \(^{15}NO\(_3\) or PO\(_4\)\(_3\).