Proton Motive Force in Washed Cells of *Rhizobium japonicum* and Bacteroids from *Glycine max*

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The components of the proton motive force (Δp), namely the membrane potential and the transmembrane pH gradient, were measured in washed cells of *Rhizobium japonicum* CC705 grown in cultures (5% O₂-95% N₂) in the presence of 10 mM KNO₃ and in bacteroids from *Glycine max*. The Δp and its components remained reasonably constant in cells as well as in bacteroids at various stages of growth. The effects of uncouplers and ATPase inhibitors on the Δp and its components were determined in both cultured cells and bacteroids. The data indicated that a respiration-driven H⁺ translocation is the source of the Δp in both cultured cells and bacteroids.

Some biochemical aspects of denitrification in *Rhizobium japonicum* have been studied (2, 5, 6, 14–20). We have shown that [¹⁵N]NO₃⁻, [¹⁵N]NO₂⁻, and N₂O were denitrified into N₂ gas by washed cells of *R. japonicum* CC705, as well as by bacteroids prepared from root nodules of *Glycine max*. Some of the [¹⁵N]NO₂ gas produced by the denitrification of [¹⁵N]NO₃⁻ in bacteroids was recycled by nitrogenase into cell nitrogen (5). One possible function for denitrification in *R. japonicum* is the conversion of NO₃⁻ and NO₂⁻, which inhibits dinitrogen fixation, into the nontoxic gases N₂O and N₂. We have also shown that ATP is synthesized during the denitrification of NO₃⁻ in cultured cells of *R. japonicum* and in bacteroids prepared from *G. max* (2).

Bacteroids derive ATP for dinitrogen reduction primarily from oxidative phosphorylation coupled to electron transport to O₂ (1). Under anaerobic conditions, *R. japonicum* bacteroids use NO₃⁻ as a terminal electron acceptor in place of O₂ (7). Although the proton motive force (Δp) has been determined in a number of bacteria (3, 4, 8, 9, 11, 15), no such information is available for *R. japonicum* grown in cultures under denitrifying conditions or in bacteroids prepared from *G. max*. Laane et al. (13) measured the Δp in bacteroids of *Rhizobium leguminosarum* in relation to nitrogenase activity, as well as the ATP-ADP ratio in the presence of valinomycin and nigericin. Gober and Kashket (7) recently reported on the magnitude of the Δp of cowpea *Rhizobium* sp. strain 32H₁⁺ in cells grown under N₂-fixing and nontoxic conditions.

*R. japonicum* CC705 cells grown under 5% O₂-95% N₂ in a medium containing 10 mM KNO₃ were harvested, washed, and suspended in 50 mM sodium phosphate buffer (pH 7.5) as described previously (2). Bacteroids from 3-week-old root nodules were prepared as described previously (2). All buffers and solutions were sparged with argon for 20 min (20 ml/min) before use to maintain anaerobic conditions. Intra- and extracellular space was determined by methods using [³H]H₂O, [¹⁴C]sucrose, and [¹⁴C]inulin (14, 15). Isotopically labeled compounds (10 µl) and NaNO₃ (10 mM) were dispensed into Eppendorf centrifuge tubes (1.5 ml) fitted with rubber septa (Sigma). The tubes, evacuated for 1 min through needles inserted into the sub-seals, were then filled with argon. After the addition of a 500-µl suspension of either washed cells of *R. japonicum* or bacteroids (treated with 10 mM EDTA in 50 mM sodium phosphate buffer [pH 7.5]) via gastight microsyringes, incubation was continued at 25°C for 10 min. Samples were centrifuged through a silicone oil mixture (0.5 ml) as described by Kashket (8). Portions of the supernatant (200 µl) were carefully removed with a pipette, and the pellets were swabbed dry with cotton buds (10) before resuspension in 200 µl of distilled water. Supernatant and pellet fractions were counted for radioactivity in a Packard Tri-Carb 460CD liquid scintillation spectrometer, as described previously (12). For membrane potential (Δψ) determinations, [³H]tetraphenylphosphonium bromide ([³H]TPP⁺ [200 nCi ml⁻¹]) was added to a cell suspension (1 to 1.5 mg [dry weight] ml⁻¹). The pH gradient (ΔpH) was determined by adding [¹⁴C]benzoic acid (200 nCi ml⁻¹) to similar cell suspensions. The final concentrations of [³H]TPP⁺ and [¹⁴C]benzoic acid were 8.5 nM and 8.8 µM, respectively. The calculations for Δψ and ΔpH were made with reference to the Nernst equation (9), after correcting for nonspecifically bound [³H]TPP⁺ and extracellular counts of [¹⁴C]benzoic acid, respectively (8).

*R. japonicum* cells grown anaerobically with KNO₃ exhibited nitrate-dependent proton ejection (16). The results presented here for cells grown in the presence of 5% O₂-95% N₂ (Fig. 1A and 1B) indicate that the intracellular pH was more alkaline than the external pH at external pH values below 8 for cultured cells grown in the presence of nitrate and below 7.5 for bacteroids. Over a range of external pH values (6 to 7.5), cultured cells or bacteroids had a limited capacity to maintain a constant internal pH. The enteric gram-negative bacteria usually maintain a constant internal pH, whereas other bacteria do not (3, 4, 7–16).

Cultured cells and bacteroids had no ΔpH (inside alkaline) at an external pH of 8.0 and 7.5, respectively (Fig. 1A and 1B). At these pH values, when the ΔpH was zero, the respiration-dependent H⁺ pump then would establish a Δp consisting entirely of Δψ of about -200 mV (cells) and -180 mV (bacteroids). The Δp in washed cultured cells remained constant (about -200 mV) when the external pH was varied from 6 to 8, but the contributions of the Δψ and ΔpH to the Δp over this range of pH values were different. In bacteroids (Fig. 1B), a small decrease in the Δp (-181 to -170 mV) was seen when the pH increased from 6 to 8. Irrespective of the stage of growth, the value for Δψ was -170 mV and the

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pH was 7.5, and 59ΔpH was 5.9 mV at pH 7. The constant Δp values (about -200 mV) are consistent with this gradient not being rate limiting for growth.

The Δp values in the absence of nitrate were -210 mV for cultured cells and -185 mV for bacteroids, and comparable values with nitrate were -200 and -178 mV. A small decrease in the Δp (-175 to -164 mV) was recorded for cultured cells when NO₃⁻ concentrations in the reaction mixtures were increased from 5 to 50 mM, whereas the Δp decreased from 35.4 to 14.1 mV (inside alkaline) (Fig. 2A). When NO₃⁻ was increased from 5 to 50 mM (Fig. 2A), a decrease in the Δp and ΔpH resulted in an overall reduction in the Δp of 32 mV (-210 to -178 mV). A decrease in the Δp, however, was more pronounced in bacteroids (-174 to -130 mV) when NO₃⁻ was increased from 5 to 50 mM, and this was accompanied by a change in the ΔpH of 11.8 mV. At 50 mM NO₃⁻, the ΔpH was completely abolished (Fig. 2B). Variations between replicate experiments were within 10%.

Sodium azide (1 mM), which inhibits nitrate reductase activity in membrane fractions of cultured cells grown in the presence of 5% O₂–95% N₂ (2), also reduced the Δp values in both cultured cells and bacteroids at higher concentrations (2.5 mM) (Table 1). Nitrate reduction occurs during both growth and assay conditions. The uncouplers carbonyl cyanide-m-chlorophenyl-hydrazone (CCCP) and 2,4-dinitrophenyl (DNP) markedly reduced the Δp and ΔpH in both cells and bacteroids. CCCP at 0.1 mM completely abolished the ΔpH, thus collapsing the Δp from 200 to 35 mV in cells and from 173 to 31 mV in bacteroids (Table 1). N,N'-dicyclohexylcarbodiimide (DCCD), a potent covalently reacting inhibitor of H⁺-ATPase of bacterial membranes, reduced the Δp in cells and bacteroids less than did CCCP and DNP, but its effect on the ΔpH was more pronounced, especially in bacteroids. At these relatively high concentrations (0.2 mM), DCCD could have nonspecific side effects, possibly involving the modification of carboxyl groups.

In cells of _R. japonicum_ and bacteroids of _G. max_, the overall behavior of the Δp and ΔpH were similar to that reported for other bacteria (12, 18). The effects of sodium azide, uncouplers, and ATPase inhibitors, which decreased the Δp in cells and bacteroids, indicated that the protons are pumped outward primarily by the proton-pumping respiratory chain.

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**LITERATURE CITED**
