THE INVOLVEMENT OF THE MEMBRANE POTENTIAL IN NITROGEN FIXATION BY BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM*

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1. Introduction

One of the major problems concerning nitrogen fixation in obligate aerobes is the generation of reducing equivalents for nitrogenase. Current evidence with *Azotobacter vinelandii* [1–5] and with pea nodule bacteroids [6–8] indicates that the generation of reducing equivalents rather than the ATP production is the rate limiting factor in the process of aerobic nitrogen fixation. It was shown that the energized state and the integrity of the cytoplasmic membrane controls the supply of reducing equivalents to nitrogenase. Recently, a proposal has been made for the electron transport to nitrogenase in *A. vinelandii* [2]. According to this hypothesis the protonmotive force across the cytoplasmic membrane, generated by respiration, drives the thermodynamically unfavourable reduction of flavodoxin to its hydroquinone form by NADH via a NADH flavodoxin oxidoreductase. This driving force, the so-called electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$), is composed of an electrical ($\Delta\Psi$) and a concentration gradient (ΔpH) across the membrane [9–11] and specific ionophores are known to dissipate either one of these gradients [12].

Here we investigated the effect of ionophores on nitrogen fixation by respiring bacteroids of *Rhizobium leguminosarum*. Evidence is presented that the membrane potential ($\Delta\Psi$) across the cytoplasmic membrane of bacteroids regulates the generation of reducing equivalents for nitrogenase.

Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; BSA, bovine serum albumin; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol; Ph_4P^+, tetraphenylphosphoniumbromide

2. Materials and methods

2.1. *Bacteroid suspensions and myoglobin preparation*

Bacteroids of *R. leguminosarum* (strain PRE) were produced under controlled conditions on peas (*Pisum sativum* L. cv. Rondo) and isolated from the root nodules as in [6]. Bacteroids were washed twice by centrifugation in a medium containing 25 mM Tes/NaOH, 2.0 mM $MgCl_2$, 1.0 mM KCl, 0.3 M sucrose and 2.5% fatty acid-free BSA, final pH 7.4. They were finally resuspended and stored at 0°C at ~60 mg protein/ml in the same medium except that BSA was omitted.

Myoglobin was oxygenated as described [13].

2.2. *Analytical methods*

The nitrogenase activity (acetylene reduction) of bacteroids was measured in the assay system of [6,8]. In all experiments the oxygen supply was sufficient to allow maximal rates of acetylene reduction (see [6], fig.3). The standard incubation buffer contained: 25 mM Tes/NaOH, 2.0 mM $MgCl_2$; 1.0 mM KCl, 0.3 M sucrose and 20 mM sodium succinate, final pH 7.4 or 6.7. When the rate of acetylene reduction was constant ionophores were added and incubation was continued for ⩾8 min.

Flow dialysis experiments were conducted aerobically at 25°C as in [14]. The upper and lower chambers were separated by dialysis tubing (Vishking; pore

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diam. 2.4 nm). The upper chamber (0.85 ml) contained the standard buffer at a given pH and 230 μM oxygenated myoglobin. Reactions were started by the addition of cells (0.05 ml), since the endogeneous rate of respiration was rather high. Further additions were made as indicated. The same buffer without myoglobin was pumped through the lower chamber at 3 ml/min. Fractions (1.0 ml) were collected and assayed for radioactivity. The experiments were performed under optimal conditions for nitrogen fixation. This was done by flushing pure oxygen over the medium in the upper chamber and by adjusting the stirring speed or the amount of cells. ΔpH and the ΔΨ were calculated from the steady-state concentration gradients of acetate and tetraphenylphosphoniumbromide (Ph_4P^+) [14,15], respectively. The internal volume of the bacteroid was determined as in [16] and was calculated to be 5.2 μl/mg cell protein. Flow dialysis figures are presented as in [17].

The intracellular levels of ATP, ADP and AMP were determined as in [1].

Protein concentrations were determined by the biuret method.

[U-^{14}C]Acetic acid (60 Ci/mol) was obtained from the Radiochemical Centre (Amersham, Buckinghamshire). [U-^{3}H]Tetraphenylphosphoniumbromide (113.5 Ci/mol) was a generous gift of Dr H. R. Kaback.

3. Results and discussion

To investigate the role of $\Delta\tilde{\mu}_{H^+}$ or its components on the process of nitrogen fixation valinomycin and nigericin were used. Valinomycin, facilitates the electrogenic movement of K^+ across the membrane, causing dissipation of the membrane potential (ΔΨ) and, in some instances, reciprocal enhancement of the transmembrane pH difference (ΔpH). Nigericin, on the other hand, facilitates the electroneutral exchange of H^+ mainly for K^+, causing dissipation of the ΔpH and, in some cases, reciprocal enhancement of the ΔΨ [12,14,15,18].

Figure 1 illustrates the effect of valinomycin (panels A) and nigericin (panels B) on the nitrogenase activity and the ATP/ADP ratio (I) as well as on $\Delta\tilde{\mu}_{H^+}$, ΔΨ, ΔpH and the intracellular pH (II) in bacteroids of *R. leguminosarum*. The panels IIA and B compile the

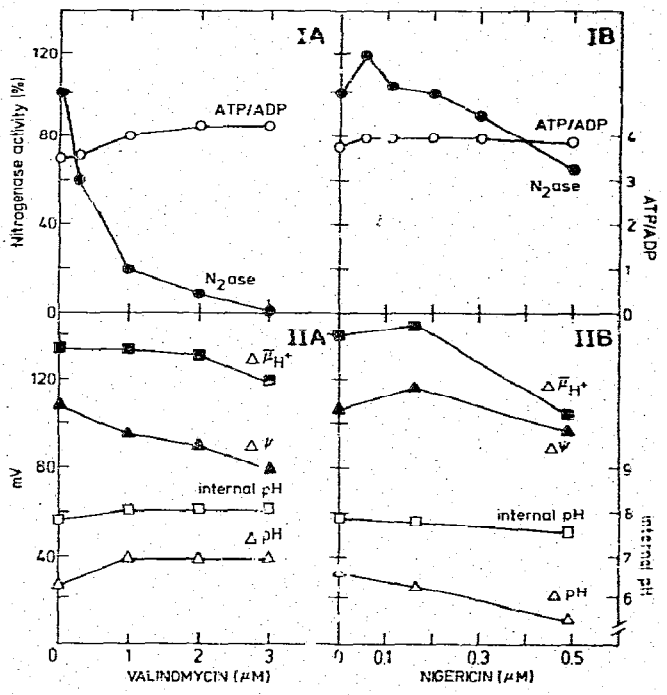

Fig.1. Effect of valinomycin (panels A) and nigericin (panels B) on nitrogenase activity (N_2ase), ATP/ADP ratio (I), $\Delta\tilde{\mu}_{H^+}$, ΔΨ, ΔpH and internal pH (II) in bacteroids of *R. leguminosarum*. Bacteroids were isolated and experiments were performed at an external pH 7.4 as described in section 2. To the standard incubation buffer bacteroids and myoglobin were added to final conc. 4.0 mg protein/ml and 230 μM, respectively. Data in panels II were obtained from flow dialysis as presented and described in fig.2.

data obtained from flow dialysis experiments as presented in fig.2. Addition of increasing amounts of valinomycin resulted in a decrease of the nitrogenase activity and an increase of the ATP/ADP ratio, while the rate of succinate oxidation was hardly influenced. At low concentration (≤2 μM) valinomycin hardly affected the $\Delta\tilde{\mu}_{H^+}$, since the decrease in ΔΨ was compensated by an increase in ΔpH. This phenomenon is quite similar to that observed in membrane vesicles of *Escherichia coli* [14]. Under optimal conditions for nitrogen fixation the ΔpH was calculated to be 0.45 pH units. The intracellular pH is therefore ~7.9. As the concentration of valinomycin was increased, the internal pH rose to a fairly constant level of ~8.1.

Nigericin at <0.2 μM stimulated the nitrogenase activity significantly, while at higher concentrations

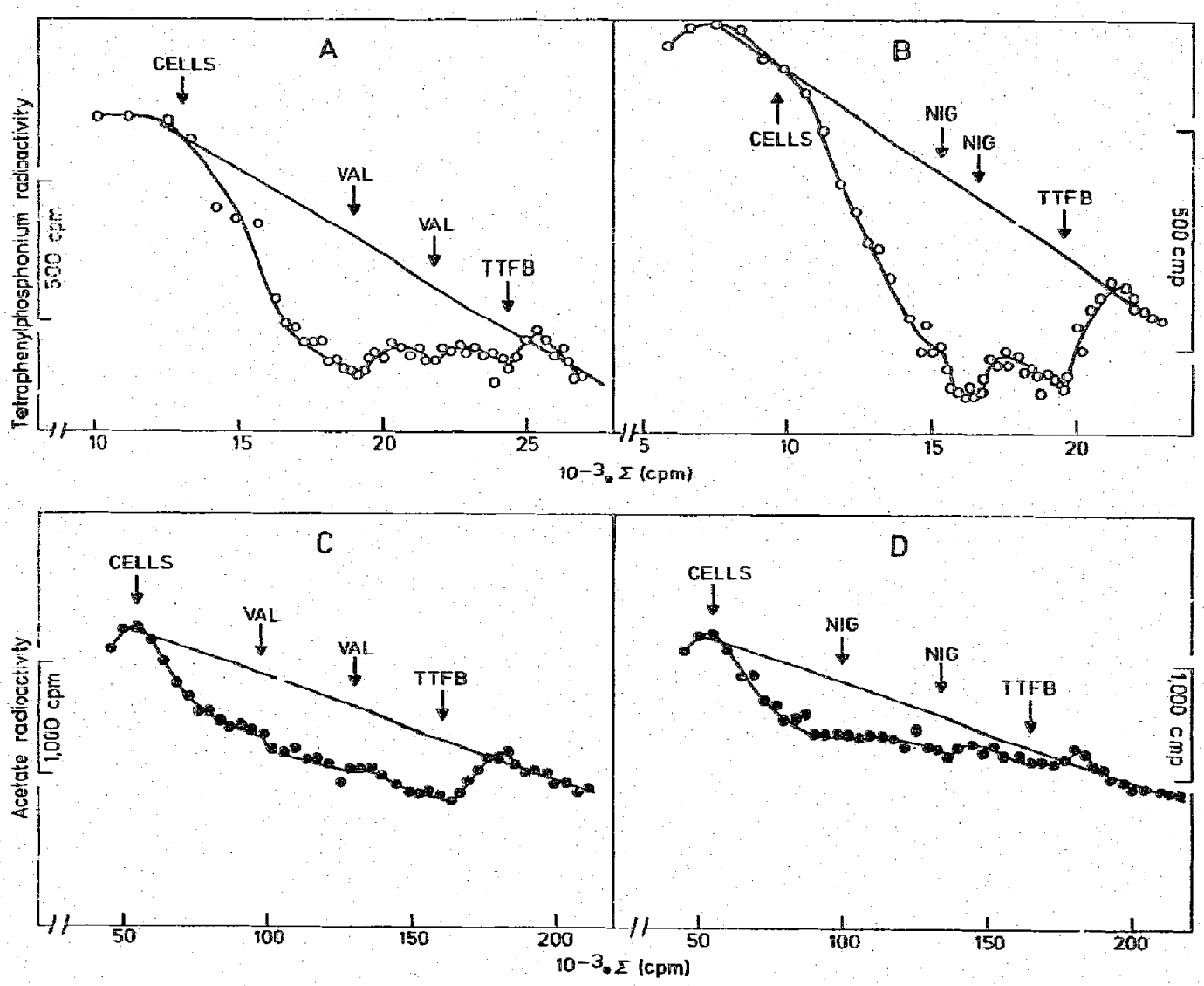

Fig.2. Effect of valinomycin and nigericin on uptake of tetraphenylphosphonium (A,B) and acetate (C,D) by bacteroids of *R. leguminosarum*, as determined by flow dialysis. Bacteroids were isolated and experiments were performed at an external pH 7.4 as described in section 2. Oxygenated myoglobin, [^{3}H]tetraphenylphosphonium and [^{14}C]acetate were added to the upper chamber at final conc. 230 μM, 24 μM and 30 μM, respectively. Reactions were started by the addition of 0.05 ml cells, final conc. 5.3 mg/ml. Valinomycin (VAL), nigericin (NIG) and uncoupler (TTFB) were added at the times indicated by arrows up to final conc. 1 μM (first addition, 2 μM second addition), 0.14 μM (first addition, 0.35 μM second addition) and 10 μM, respectively. Figures are presented as described [17].

the rate of acetylene reduction was inhibited (panel 1B). The ATP/ADP ratio and the rate or respiration remained unchanged within the concentration range used. The increase in nitrogenase activity was accompanied by an increase in $\Delta\Psi$, while the transmembrane pH difference as well as the internal pH decreased.

Nigericin at >0.2 μM had an uncoupling effect, as can be seen by the decline in $\Delta\tilde{\mu}_{H^+}$.

In vivo nitrogenase functions only when cells generate enough energy in the form of ATP and reducing equivalents with a sufficiently low potential. Since the intracellular level of ATP (expressed in the ATP/ADP

ratio) is hardly influenced by the addition of valinomycin or nigericin, it is clear that the decline or stimulation of the nitrogenase activity by the addition of these ionophores is not due to a decreased supply of ATP to nitrogenase. Furthermore, our results indicate that the decline in nitrogenase activity is not caused by a decrease in $\Delta\tilde{\mu}_{H^+}$. In our opinion, two possible explanations remain which can account for the observed effects of these ionophores on nitrogen fixation by bacteroids:

1. By an effect of the internal pH on nitrogenase itself or on the electron transport system to nitrogenase;
2. By a direct effect of $\Delta\Psi$ on the electron transport system to nitrogenase.

Figure 3 shows the relationship between the nitrogenase activity and $\Delta\tilde{\mu}_{H^+}$ (A), ΔpH (B) and $\Delta\Psi$ (C). In addition the internal pH values are given. It should be noted that the nitrogenase activity resembles the flow of reducing equivalents to nitrogenase, since the ATP/ADP ratios under the applied experimental conditions are hardly influenced. This figure also contains the results obtained from experiments performed at an external pH 6.7 instead of 7.4 (open symbols). At an external pH 6.7 the nitrogenase activity, at a fixed oxygen supply, was still 90% of the activity at 7.4. At pH <6.7 the nitrogenase activity declined dramatically. Like in several bacteria [19–21] the ΔpH varied markedly with external pH. Despite an increase in ΔpH, at lower external pH, the internal pH and the $\Delta\Psi$ remained fairly constant. Intracellular pH values at extracellular pH 6.7–7.4 were almost constant at 7.65–7.85. Consequently, $\Delta\tilde{\mu}_{H^+}$ rose from about −130 mV at pH 7.4 to −160 mV at pH 6.7, while the nitrogenase activity was 10% lower and the ATP/ADP ratio hardly changed (not shown). The effect of valinomycin on the nitrogenase activity appeared to be independent of the external pH. At 1 μM the nitrogenase activity dropped in both cases to 20%. However, nigericin was far more effective at pH 6.7 than at pH 7.4. This observation is consistent with the finding that at a higher external pH the ΔpH is only small. At an external pH 6.7 nigericin at 0.014 μM stimulated the nitrogenase activity maximally by 40%, while at pH 7.4 more nigericin (0.06 μM) was necessary for maximal stimulation of the nitrogenase activity (20%).

Figure 3 clearly demonstrates that there is no correlation between the flow of reducing equivalents to nitrogenase and $\Delta\tilde{\mu}_{H^+}$ or ΔpH in bacteroids of *R. leguminosarum*. The nitrogenase activity changes markedly, while $\Delta\tilde{\mu}_{H^+}$ or ΔpH are not influenced. On the other hand, there is a clear relationship between the nitrogenase activity and $\Delta\Psi$. All data, whether they are obtained from different experiments performed at different external pH or in the presence of ionophore fit into this relationship. Since the internal pH does not change significantly under these conditions (fig.3C), it is clear that electron transport to nitrogenase is regulated by the $\Delta\Psi$ and extremely sensitive

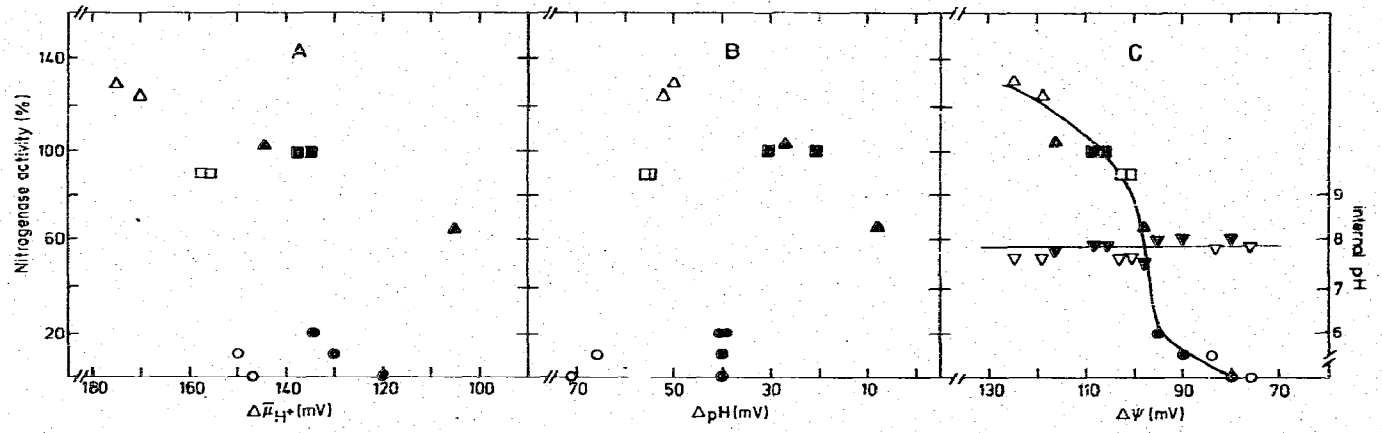

Fig.3. Relationship between nitrogenase activity and $\Delta\tilde{\mu}_{H^+}$ (A), ΔpH (B), $\Delta\Psi$ (C) in bacteroids of *R. leguminosarum*. Data at an external pH 7.4 (dark symbols) are obtained from experiments presented in fig.1. Open symbols represent the results obtained at an external pH 6.7 (see text). 100% nitrogenase activity ≃20 nmol ethylene produced .min^{-1} .mg bacteroid protein^{-1} at pH 7.4. (□,■) Without addition; (△,▲) with nigericin; (○,●) with valinomycin; (▽,▼) internal pH.

towards changes in $\Delta\Psi$. At $\Delta\Psi < -80$ mV no functional reducing equivalents are generated, while at > -120 mV the machinery which produces reducing equivalents seems to function maximally. Especially small changes in $\Delta\Psi$ centered around −100 mV are accompanied by marked changes in the flow of reducing equivalents to nitrogenase.

We also studied the effect of valinomycin and nigericin on the ATP/ADP ratio and on the nitrogenase activity of the free-living nitrogen fixer *A. vinelandii* (not shown). Valinomycin and nigericin were found to be very active in *A. vinelandii* provided cells were pretreated with EDTA [22]. However, both the rate of acetylene reduction as well as the ATP/ADP ratio dropped considerably when low concentrations of valinomycin or nigericin were added. *A. vinelandii* accumulates K^+ actively during respiration and K^+ is required for an optimal functioning metabolism [23]. For example, the rate of respiration decreased ~2-fold when K^+ was omitted from the medium. Furthermore, without K^+ little nitrogenase activity could be observed. Since valinomycin and nigericin disturb the K^+ gradient in vivo, it seems reasonable to suggest that they also influence essential processes for nitrogen fixation. We observed no influence of K^+ on the metabolism of *R. leguminosarum* bacteroids. In our opinion, bacteroids do not need a K^+-accumulating system, since bacteroids are localized within the plant host cell which already contains a relatively high concentration of K^+.

At this stage of investigation the mechanism of a $\Delta\Psi$-dependent formation of reducing equivalents in bacteroids of *R. leguminosarum* is not understood. Work is in progress to characterize the function of the $\Delta\Psi$ in the generation of reducing equivalents with a sufficiently low potential for nitrogenase in isolated membrane vesicles of *R. leguminosarum* bacteroids.

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