Effects of K⁺ on the Proton Motive Force of Bradyrhizobium sp. Strain 32H1

JAMES W. GOBER AND EVA R. KASHKET

Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118

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In previous studies, respiring Bradyrhizobium sp. strain 32H1 cells grown under 0.2% O₂, conditions that derepress N₂ fixation, were found to have a low proton motive force of < -121 mV, because of a low membrane potential (ΔΨ). In contrast, cells grown under 21% O₂, which do not fix N₂, had high proton motive force values of > -175 mV or more, which are typical of respiring bacteria, because of high ΔΨ values. In the present study, we found that a ΔΨ of 0 mV in respiring cells requires growth in relatively high-[K⁺] media (8 mM), low O₂ tension, and high internal [K⁺]. When low-[O₂], high-[K⁺]-grown cells were partially depleted of K⁺, the ΔΨ was high. When cells were grown under 21% O₂ or in medium low in K⁺ (50 μM K⁺), the ΔΨ was again high. The transmembrane pH gradient was affected only slightly by varying the growth or assay conditions. In addition, low-[O₂], high-[K⁺]-grown cells had a greater proton permeability than did high-[O₂]-grown cells. To explain these findings, we postulate that cells grown under conditions that derepress N₂ fixation contain an electrogenic K⁺/H⁺ antiporter that is responsible for the dissipation of the ΔΨ. The consequence of this alteration in K⁺ cycling is rerouting of proton circuits so that the putative antiporter becomes the major pathway for H⁺ influx, rather than the H⁺-ATP synthase.

The magnitude of the proton motive force (Δp) of cowpea Bradyrhizobium sp. strain 32H1 is dependent on the growth conditions (7). Cells grown under atmospheric O₂ (high [O₂]), conditions that repress N₂ fixation, had a Δp of -185 mV or more, negative and alkaline inside. A Δp of this magnitude is typical of respiring bacteria (for a review, see reference 12). In contrast, cells grown under 0.2% O₂ (low [O₂]), conditions permitting N₂ fixation, had a Δp of -121 mV or less. The low Δp of low-[O₂]-grown cells was due to a low transmembrane electrical gradient (ΔΨ), whereas the pH gradient (ΔpH) was similar to that of high-[O₂]-grown cells. We hypothesized that the low ΔΨ was the result of an increased electrogenic H⁺-linked flux of another ion in cells grown under low O₂ tensions. The most obvious candidate was K⁺, because this cation has been shown to convert ΔΨ into ΔpH in a number of bacterial systems (1, 8, 13–15, 20, 24, 26). K⁺ ions have been assigned a role in regulating the internal pH in Escherichia coli and other bacterial cells, particularly at alkaline pH (2–4, 20, 22, 24, 26). K⁺ has also been shown to be required for maintenance of a high ΔΨ in E. coli incubated in Tris buffer at alkaline pH (11).

In this report, we show that the ΔΨ of Bradyrhizobial is low or nonexistent only in the cells grown under low-[O₂], high-[K⁺] conditions and tested with high internal and low external [K⁺]. Growth of the cells under high-[O₂] atmospheres or in low-[K⁺] media, or tested after partial depletion of internal K⁺, resulted in a high ΔΨ. We postulate the existence in cells showing a low ΔΨ of an electrogenic K⁺/H⁺ antiporter, the NKH system.

MATERIALS AND METHODS

Growth of cells. Bradyrhizobium sp. (Vigna) strain 32H1 (cowpea Bradyrhizobium sp. strain 32H1) cells were grown in either 21% (high [O₂]) O₂ or 0.2% (low [O₂]) O₂, as described previously (6). The media were used as a high-[K⁺] medium (containing approximately 8 mM K⁺), which is the medium described previously (7), or a low-[K⁺] medium, which has the same composition as the high-[K⁺] medium, except that NaH₂PO₄ (1.02 g/liter) was substituted for KH₂PO₄ and KCl was added to a final concentration of approximately 50 μM.

Preparation of cell suspensions. The Δp assays were performed with nongrowing, respiring cells harvested from early-stationary-phase (4 day) cultures, washed twice with 0.1 M 3-N-(morpholino)propanesulfonic acid (MOPS) adjusted to pH 7.0 with Tris base (MOPS-Tris buffer), and suspended in one of the following buffers: 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) adjusted to pH 6.0 with Tris base (MES-Tris buffer), MOPS-Tris buffer, pH 7.0, or 0.1 M N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid (EPSS) adjusted to pH 8.0 with Tris base (EPSS-Tris buffer). The buffers were supplemented with 12.3 mM sodium succinate and 6.6 mM d-arabinose, as well as 5 mM KCl, as indicated.

K⁺ loading and depletion. For K⁺ loading, cells were harvested, suspended in MOPS-Tris buffer, pH 7.0, and incubated for 30 min at 28°C with 6.6 mM d-arabinose, 12.3 mM sodium succinate, and 10 mM KCl. The cells were then centrifuged, washed twice with MOPS-Tris buffer, pH 7.0, and resuspended in the buffers described above. For K⁺ depletion, the cells were harvested, washed twice, resuspended in 50 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), adjusted to pH 8.5 with Tris base (TAPS-Tris buffer), shaken for 15 min at 28°C in 21% O₂, recentrifuged, and then resuspended in the buffers described above.

Measurements of [K⁺] and Δp. Suspended cells were assayed for K⁺ content and Δp at a final concentration of 0.38 to 0.57 mg (dry weight) per ml, equal to an optical density at 625 nm of 1.0 to 1.5. For [K⁺] measurements, the cells were collected by centrifugation through silicone oil, permeabilized with acetone, and assayed by flame photometry as described previously (13). Measurements of ΔpH and ΔΨ were carried out as described previously (7).

Proton permeability. The passive permeability of the cell membrane to H⁺ was measured with deenergized, anaerobic cells by the method of Mitchell and co-workers (18, 23). The
cells were harvested in the early stationary phase and washed three times and suspended in 3 mM MOPS-50 mM KCl adjusted to pH 6.3 with KOH. 2,4-Dinitrophenol adjusted to pH 7.2 with Tris base was added to a final concentration of 1 mM, and the cell suspension was incubated with rapid shaking overnight at 28°C in the O2 concentration used during growth. The cells were then harvested, washed six times to remove the 2,4-dinitrophenol, resuspended in the buffer described above to a final cell density of 2.3 to 3.8 mg (dry weight) per ml, and placed in a water-jacketed electrode chamber for external pH measurements. The reaction mixture (4.0-ml total volume) was mixed continuously and sparged with humidified, O2-free N2. After the addition of cells, carbonic anhydrase (2 mg/ml), and KSCN (50 mM) to the buffer, the incubation mixture was allowed to equilibrate at pH 6.0 to 6.3 for about 30 min at 28°C. For low-[K+] experiments, NaOH-neutralized MOPS buffer and NaSCN were used. A known amount of anaerobic HCl was added to start the reaction, which was monitored by recording the pH of the medium. The internal, external, and total buffering powers and the conductance to H+ (Cm) were calculated from the rates of realalkalinization of the medium (18, 23).

Rate of respiration. The rate of O2 uptake was measured at 28°C with a Clark-type electrode (Rank Bros., Bottisham, Cambridge, England) either with K+-depleted cells or with 5 mM KCl added, in MOPS-Tris buffer, pH 7.0, supplemented with 6.6 mM d-arabinose and 12.3 mM sodium succinate.

Materials. The radioactive Δp probes, [7,14C]benzoate, [3H]tetraphenyl phosphonium, and [3H]polyethylene glycol, were bought from Dupont-New England Nuclear Corp., Boston, Mass. The other chemicals were of reagent grade and are available commercially.

RESULTS

Effect of pH on the internal [K +]. The internal [K+] of respiring bradyrhizobia was found to be influenced significantly by both the external [K+] and the external pH. Respiring cells incubated in buffer accumulated K+ at neutral pH and extruded it at alkaline pH. For example, cells grown under low [O2], harvested, washed, and resuspended in MOPS-Tris buffer, pH 7.0, containing 16 μM K+ maintained an internal [K+] of 98 to 112 mM for at least 30 min (Fig. 1). When 5.8 mM KCl was added at the beginning of the experiment, the internal [K+] increased to approximately 170 mM after 30 min of incubation. In contrast, the internal [K+] of these cells decreased to 45 to 53 mM after 30 min of incubation at pH 8.0 in EPPS-Tris buffer containing either 30 μM or 5.4 mM K+ . The efflux of K+ at high pH, particularly in the presence of permeant amines, has been observed in other bacteria (11, 19, 25-27). Net efflux of K+ at high pH occurred in these bradyrhizobia grown under any of the following conditions used in this study: in low (0.2%) or high (21%) [O2] atmospheres and in media containing either low (50 μM) or high (8 mM) [K+] (data not shown). K+ efflux at high pH was seen also in growing cells when the medium reached pH 8.0 or higher (data not shown). Therefore, K+-loaded cells were prepared routinely by incubation in buffer at pH 7.0 in the presence of 10 mM K+, and K+-depleted cells were prepared by incubation at pH 8.5 in low-[K+] buffers.

Effect of internal and external [K+] on Δp. In previous experiments, we found that cells grown under conditions conducive to N2 fixation (low [O2], high [K+]) had a low Δp when tested in buffers containing 5 mM KCl (7). Cells grown under high [O2], which represses N2 fixation, but otherwise treated identically, had a high Δp. To separate the effects of [O2] and [K+], we first examined the effects of varying the internal [K+] on Δp. Cells grown under low-[O2], high-[K+] conditions were either partially depleted of K+ by incubation at high pH or loaded with K+ by incubation at pH 7 (see Materials and Methods). The cells were then allowed to respire at low external [K+] in buffers at pH 6.1, 7, or 8. In partially K+-depleted cells, the Δp was relatively high, ranging from −168 mV at pH 6.1 to −146 mV at pH 8.0 (Fig. 2A). The Δp ranged from −102 mV at pH 6.1 to −117 mV at pH 8.0. The 59ΔpH was 67 mV at pH 6.1, 31 mV at pH 7, and 22 mV at pH 8.0; the internal pH thus ranged from pH 7.2 to 8.4.

In contrast to K+-depleted cells, K+-loaded cells suspended in low-[K+] buffers had no measurable Δp at all medium pHs tested (Fig. 2B). The lack of uptake of 85CN− by these cells indicated that they did not have a Δp of an inside-positive polarity (data not shown). The 59ΔpH values ranged from 98 mV at pH 6.1 to 35 mV at pH 8.1, resulting in an internal pH ranging from 7.8 at pH 6.1 to 8.7 at pH 8.0. The internal pH of K+-loaded cells was thus slightly, but significantly, more alkaline than that of K+-depleted cells. Because of the absence of a Δp, the Δp of K+-loaded cells was low, ranging from −98 mV at pH 6.1 to −31 mV at pH 8.0. It can be noted that, at pH 6.1 and 7, the internal [K+] was clearly lower in the partially K+-depleted cells than in the K+-loaded cells. At pH 8.0, however, the internal [K+] was low in both K+-depleted and -loaded cells (29 and 21 mM, respectively). Net K+ efflux probably took place during the assay period at pH 8, as suggested by the significantly
higher external [K\(^+\)] of K\(^+\)-loaded cells at pH 8 (108 \(\mu\)M) compared with the other assay conditions (<26 \(\mu\)M).

As expected from reports on other bacteria, addition of K\(^+\) to K\(^+\)-depleted, low-[O\(_2\)], high-[K\(^+\)]-grown bradyrhizobia resulted in the conversion of \(\Delta\psi\) into \(\Delta\phi\) (Table 1). K\(^+\)-depleted cells incubated at pH 6.0 had a 59\(\Delta\phi\) of 67 mV and a \(\Delta\phi\) of -76 mV, equivalent to a \(\Delta\phi\) of -143 mV. KCl (50 mM) was then added; 30 s later, the \(\Delta\phi\) was reduced to < -34 mV and was completely dissipated by 1.5 min. The internal pH rose from 7.15 to 7.57 over 15 min of incubation. The same phenomenon was observed in cells incubated at pH 7.0; the \(\Delta\phi\) was reduced by KCl within 30 s from -129 mV to 0 mV, while the internal pH rose from 7.70 to 8.44 over 15 min of incubation.

**Effect of O\(_2\) and [K\(^+\)] during growth on \(\Delta\phi\).** Bradyrhizobia grown under atmospheric O\(_2\) and at high [K\(^+\)] did not exhibit the effect of K\(^+\) on \(\Delta\phi\), and the \(\Delta\phi\) was high (>-171 mV) under all conditions tested (Fig. 3). Whether the cells were depleted of K\(^+\) (Fig. 3A) or loaded with K\(^+\) (Fig. 3B), the \(\Delta\phi\) was high (-148 to -160 mV) at pH 6.1 to 8. The 59\(\Delta\phi\) ranged from 54 to 11 mV at pH 6.1 to 8, giving an internal pH of 7.1 to 8.2 for the K\(^+\)-depleted cells and 7.4 to 8.4 for the K\(^+\)-loaded cells at pH 6.1 to 8. Thus, the \(\Delta\phi\) values ranged from -202 to -171 mV for K\(^+\)-depleted cells) and from -210 to -182 mV (for K\(^+\)-loaded cells) at pH 6.1 to 8.

Cells grown under low [O\(_2\)] and low [K\(^+\)] had high \(\Delta\phi\) values under all conditions tested (Fig. 4). Whether K\(^+\)-depleted or -loaded, at pH 6.2 to 8.0 the low-[K\(^+\)]-grown cells had a \(\Delta\phi\) of > -121 mV and a \(\Delta\phi\) for the usual magnitude, with a resulting \(\Delta\phi\) of > -164 mV. In addition, cells grown under high-[O\(_2\)], low-[K\(^+\)] conditions also had high \(\Delta\phi\) values (> -159 mV) and high \(\Delta\phi\) values (> -209 mV) in media of pH 6.2 to 8.0 (data not shown).

Finally, when the same assay conditions as those described previously were used (7), i.e., measurements of \(\Delta\phi\), in buffers containing 5 mM KCl, the low-[O\(_2\)], low-[K\(^+\)]-grown cells had a \(\Delta\phi\) ranging from -16 to -88 mV at pH 6.2 to 8. Thus, the \(\Delta\phi\) was -114 mV at these pHs, higher in magnitude than the \(\Delta\phi\) seen with an external [K\(^+\)] of <108 \(\mu\)M (Fig. 2B). Clearly, high internal [K\(^+\)] and low external [K\(^+\)] during the assays were the conditions that resulted in the lowest \(\Delta\phi\) values observed with low-[O\(_2\)], high-[K\(^+\)]-grown cells.

**Effect of K\(^+\) on the proton conductance.** Since the [K\(^+\)] of the growth medium and the intracellular [K\(^+\)] had such a pronounced effect on the magnitude of the \(\Delta\phi\), the most obvious possibility was that an electrolyte K\(^+\)/H\(^+\)
antipporter is involved in this phenomenon. Therefore, we compared the passive proton conductance of the cell membrane in the presence of K+ or Na+ as counterion. Cells grown under various [O2] and [K+] conditions were incubated under strictly anaerobic conditions to prevent respiration-effected H+ efflux. The rate of realalkalinization of the medium after an acid pulse, which reflects the rate of H+ influx, was measured (Table 2). These proton conductance values were similar to those of other bacteria (e.g., 17, 23). Low-[O2], high-[K+] grown cells had a proton conductance 2-5-fold greater in the presence of K+ than in the presence of Na+. [O2] affected the proton permeability, as growth in low [O2] resulted in a higher proton permeability than growth in high [O2]. However, cells grown in low-[K+] media had a relatively high proton permeability whether grown under low or high O2 tensions.

DISCUSSION

The main finding of these studies is that K+ ions, as well as the O2 tension during growth, regulate the magnitude of the Δψ, and hence the Δp, of Bradyrhizobium sp. strain 32H1. Cells grown under low-[O2], high-[K+] conditions, which derepress N2 fixation, had no Δψ when assayed in low-[K+] media. The Δψ was significantly greater in cells grown or assayed under other conditions of [O2] or [K+]. Bradyrhizobia grown in high-[K+] media take up K+ ions by one or more K+ transport systems, analogous to the systems of other bacteria (5, 9, 10, 16). In cells grown at low [K+], whether in high or low [O2], an additional K+ uptake system would probably be derepressed, analogous to the system described in Rhizobium sp. strain UMKL20 (16) and the repressible Kdp system of E. coli (5). Such an uptake system in the bradyrhizobia, functioning as a K+ H+ symporter, would be consistent with the relatively high proton conductance seen in cells grown in low-[K+] media. Proton permeation by this route would not be distinguishable from H+ influx via a putative K+/H+ antipporter under the experimental conditions used, because K+ ions would have equilibrated across the cell membrane during the preincubation period of the nonmetabolizing cells and thus would be present inside the cells.

The effect of K+ cycling on the internal pH of the bradyrhizobia was similar to that observed in other organisms. In cells depleted of K+, the internal pH was slightly lower than that of cells loaded with K+, and the addition of K+ to K+-depleted cells resulted in a gradual rise in the internal pH. Presumably, uptake of K+ allows the respiratory chain to pump out more H+ by partially dissipating the Δp and thus decreasing the reentry of H+ ions, which is the mechanism by which K+ is envisioned as converting Δψ to Δp (1, 24). This phenomenon has been demonstrated in other bacteria incubated at an external pH more acidic than the internal pH; the magnitude of the Δp was thus dependent on the K+ content of the incubating medium (1, 13-15, 20, 26). Consistent with this idea is the finding that addition of 5 mM KCl stimulated respiration by low-[O2], high-[K+] grown bradyrhizobia partially depleted of K+ (data not shown). Stimulation of respiration by K+ has been reported for E. coli cells (1, 11, 21).

At a high external pH, there was net efflux of internal K+ from the bradyrhizobia, as has been observed in other organisms (11, 19, 25, 27). This effect most likely is due to a K+H+ antipporter with a high pH optimum, such as the KHA system of E. coli (3, 4, 22). K+ cycling by this carrier is thought to result in acidification of the cytoplasm at an alkaline external pH and thus has been implicated in pH homeostasis in that organism. In contrast to the gram-negative enteric organisms, bradyrhizobia maintain an internal pH which is always more alkaline than the medium, but not a constant one. A K+H+ antipporter similar to the KHA system of E. coli, although it does not cause net acidification of the cytoplasm at an alkaline external pH, may prevent excessive alkalization of the cytoplasm by respiration-driven H+ extrusion.

To explain our present findings on the low Δψ of low-[O2], high-[K+] grown bradyrhizobia, we postulate a change in K+ cycling due to the activity of an electrogenic K+H+ antipporter, the NKH system. This antipporter is envisioned as coupling the exit of, for example, one K+ ion to the entry of two H+ ions, thereby dissipating the Δp generated by respiration-driven H+ efflux. Such antipporter activity is consistent with the requirement for low Δψ values of relatively high internal and low external [K+]. Moreover, consistent with a putative NKH system is the greater proton

Table 2. Proton conductance of Bradyrhizobium sp. strain 32H1

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Cationb</th>
<th>Proton conductance (nmol H+ sec-1 at pH 7.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low [O2], high [K+]</td>
<td>Na+</td>
<td>165 ± 9 (31)</td>
</tr>
<tr>
<td>Low [O2], high [K+]</td>
<td>K+</td>
<td>411 ± 25 (53)</td>
</tr>
<tr>
<td>High [O2], high [K+]</td>
<td>K+</td>
<td>143 ± 15 (28)</td>
</tr>
<tr>
<td>Low [O2], low [K+]</td>
<td>K+</td>
<td>356 ± 20 (41)</td>
</tr>
<tr>
<td>High [O2], low [K+]</td>
<td>K+</td>
<td>306 ± 24 (43)</td>
</tr>
</tbody>
</table>

a The experiments were carried out as described in the text.
b The assay mixture contained either 100 mM Na+ or 100 mM K+.

The values shown are the mean ± standard error of the mean of 28 to 53 assays (numbers in parentheses).
permeability seen in cells equilibrated in KCl than in NaCl and of low-[O$_2$]-grown cells than of high-[O$_2$]-grown cells.

The putative NKH system was expressed only in cells grown under O$_2$ tensions that also derepress N$_2$ fixation (7) and an ammonium transport system (6). K$^+$ and O$_2$ tension apparently acted as coregulators, since growth under high (21%)-[O$_2$] atmospheres or in low (ca. 50 μM)-[K$^+$] media failed to derepress the putative K$^+$/H$^+$ antiporter, as seen in the high ΔpH of these cells.

The consequence of the postulated NKH activity is re-routing of proton circuits. When the NKH system is operational, as in K$^+$-loaded cells, it becomes the major proton sink. This results in a low transmembrane proton electrochemical gradient, as well as a high H$^+$/ATP ratio of 3.8 in respiring cells (7). When cells containing the NKH system are depleted of K$^+$, however, they maintain a proton gradient as large as that of cells grown under low-[K$^+$] or high-[O$_2$]-containing conditions or of other respiring bacteria. In this case, the major route of H$^+$ influx presumably occurs through the H$^+$/ATPase, giving an apparent H$^+$/ATP ratio of 2 in respiring cells (7). Thus, in this organism, the alteration in K$^+$ cycling results in a significant modulation of the ΔpH, not just the interconversion of its components. It is also of interest that bradyrhizobia grown under conditions that derepress nitrogenase possess a low bulk proton gradient. Presumably, bacteroids in the plant cytosol exist in a medium containing at least millimolar K$^+$ concentrations, which would suggest that the NKH system is present and active in bacteroids.

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**LITERATURE CITED**


