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In Vivo ^{23}Na Nuclear Magnetic Resonance Study of Maintenance of a Sodium Gradient in the Ruminal Bacterium *Fibrobacter succinogenes* S85

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Sodium gradients (ΔpNa) were measured in resting cells of *Fibrobacter succinogenes* by in vivo ^{23}Na nuclear magnetic resonance using Tm(DOTP)$^{5-}$ [thulium(III) 1,4,7,10-tetraazacyclododecane-*N*′,*N*″,*N*‴-tetramethylenephosphonate] as the shift reagent. This bacterium was able to maintain a ΔpNa of −55 to −40 mV for extracellular sodium concentrations ranging from 30 to 200 mM. Depletion of Na^+ ions during the washing steps led to irreversible damage (modification of glucose metabolism and inability to maintain a sodium gradient).

In the ruminal bacterium *Fibrobacter succinogenes* S85, glucose and cellobiose uptake was shown to be driven by an artificial electrical gradient (ΔΨ) or sodium gradient (ΔpNa) in de-energized cells (5), suggesting a Na^+ cotransport of these sugars that could explain the requirement of sodium for growth of the bacterium (2, 7, 10). However, the presence of a sodium transmembrane gradient has never been demonstrated in *F. succinogenes* as well as it has been demonstrated in other rumen bacteria. The objective of this work was to demonstrate and measure such a sodium gradient in *F. succinogenes* and to monitor its variation according to that of the extracellular sodium concentration.

For that purpose, an in vivo ^{23}Na nuclear magnetic resonance (NMR) methodology that distinguishes the resonances of intracellular and extracellular sodium has been used to measure sodium gradients directly on living cells. The technique consists of using shift reagents. The reagents are anionic complexes of lanthanides (Dy^{3+}, Tm^{3+}, and Tb^{3+}) whose paramagnetic properties, when the reagents are exchanged with external sodium, induce a chemical shift of external Na^+ resonance. As these reagents cannot cross the cytoplasmic membrane, the intracellular sodium is not shifted. These reagents have been widely used on various biological systems, but few studies have been performed using them with bacteria. In this work we present the application of the Tm(DOTP)$^{5-}$ complex thulium(III) 1,4,7,10-tetraazacyclododecane-*N*′,*N*″,*N*‴-tetramethylenephosphonate (1) to the study of sodium gradients in a bacterium.

F. succinogenes S85 (ATCC 19169) was grown for 15 h on a chemically defined medium (8) with 3 g of cellobiose · liter^{-1}. The cells were harvested and suspended under anaerobic conditions (8, 11) in variable sodium buffers at a concentration of 10 mg of protein · ml^{-1}. Tm(DOTP)$^{5-}$ (Macrocyclics, Richardson, Tex.) was freshly added at a final concentration of 1, 5, or 6 mM just before the experiments were performed. The bacterial suspension was transferred to 10-mm-diameter tubes in each of which was centered a capillary containing [$Na_{10}{}^{+}Dy^{3+}(PPPi)_2{}^{7-}$], used for intensity calibration. In vivo ^{23}Na NMR experiments were carried out anaerobically at 39°C with a Bruker MSL 300 spectrometer operating at 79.39 MHz. ^{23}Na NMR spectra (60°pulse: 16.5 μs; repetition time, 350 ms; 900 scans, 1K) were collected every 5 min for 15 min, and then ionophores (monensin at 90 μM and valinomycin and [carbonyl cyanide *m*-chlorophenylhydrazone] CCCP at 30 μM each) were added and three extra spectra were collected. In Fig. 1A are presented the in vivo ^{23}Na NMR spectra of *F. succinogenes* resting cells, prepared in 75 mM Na^+-containing buffer and incubated in the presence of [$Na_5{}^{+}$Tm(DOTP)$^{5-}$] with increasing concentrations of sodium (50, 75, 100, and 150 mM). Intracellular Na^+ concentrations were measured from ^{23}Na NMR spectra registered on a single bacterial sample before (Fig. 1A) and after (Fig. 1B) addition of ionophores, and they were calculated from the following equation:

$$C_{in} = (I_{in} \times I'_{ex} \times 2.2)/(I_{cap} \times I'_{in} \times 884) \quad (1)$$

where C_{in} is the intracellular Na^+ concentration; I_{cap} is the integral of the ^{23}Na NMR signal of the reference capillary, 2.2 is the calibration factor of the capillary (millimolar), 884μl is the extracellular volume of the sample (in microliters), I_{in} is the integral of intracellular ^{23}Na NMR signals in the absence of ionophores (Fig. 1A), and I'_{ex} and I'_{in} are the integrals of extracellular and intracellular ^{23}Na NMR signals, respectively, in the presence of ionophores (Fig. 1B).

The addition of ionophores allowed us to equilibrate intracellular and extracellular Na^+ concentrations and thus to avoid measurement of the intracellular volume, which is often difficult to ascertain (a detailed calculation using this equation will

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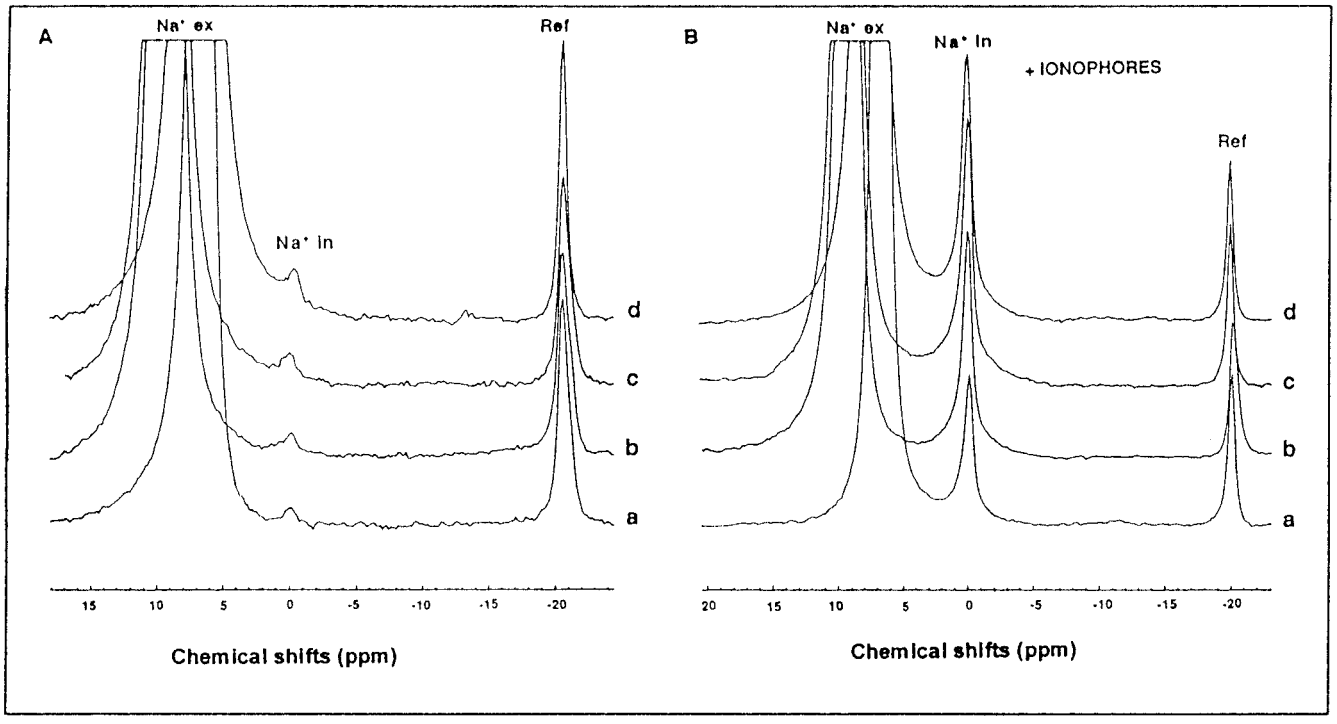

FIG. 1. In vivo ^{23}Na NMR experiments. *F. succinogenes* S85 resting cells (10 mg of protein · ml^{-1}) washed in 75 mM Na^+ solutions were incubated in buffers containing 50 mM Na^+ and 5 mM $Tm(DOTP)^{5-}$ (a), 75 mM Na^+ and 6 mM $Tm(DOTP)^{5-}$ (b), 100 mM Na^+ and 6 mM $Tm(DOTP)^{5-}$ (c), and 150 mM Na^+ and 6 mM $Tm(DOTP)^{5-}$ (d). (A) In vivo ^{23}Na NMR spectra collected after 15 min in the absence of ionophore. (B) Spectra collected after a further 15 min of incubation of the same samples in the presence of monensin (90 μM), valinomycin (30 μM), and FCCP (30 μM).

be published elsewhere). Experiments (not shown) were also performed with cells prepared in various buffers (25, 50, and 100 mM) and resuspended to reach the final external sodium concentrations of interest: 30, 50, 75, 100, 150, and 200 mM.

The values of C_{in}, calculated by using equation 1 under these differing conditions, are reported in Fig. 2. Intracellular Na^+ concentrations of *F. succinogenes* resting cells slightly increased, from about 4 to 12 mM, when extracellular sodium was increased from 30 to 100 mM. The resulting sodium gradient (ΔpNa) was calculated as $\Delta pNa = 2.3 \times RT/ZF \times \log C_{in}/C_{ex}$, where R is 8.32J · K^{-1} · mol^{-1}, T is 312 K, F is 96,500, Z is 1 (cation charge), C_{in} is the intracellular sodium concen-

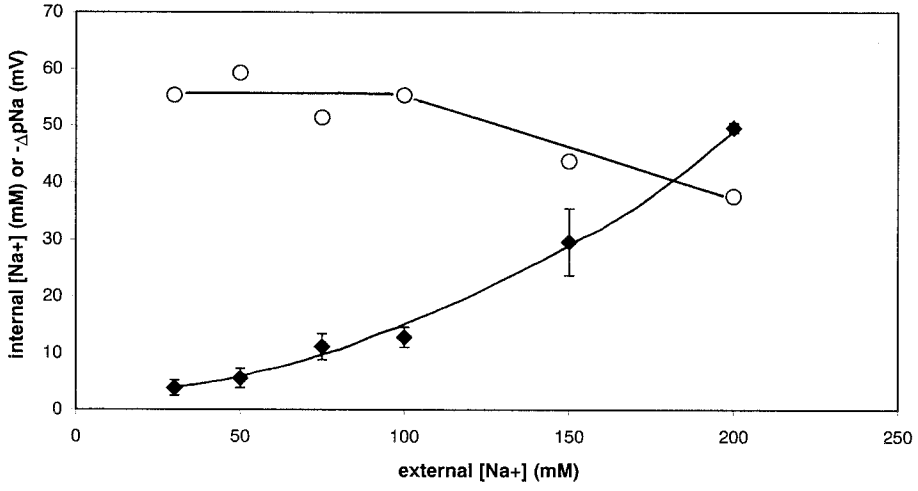

FIG. 2. Intracellular sodium concentrations (◆) measured by in vivo ^{23}Na NMR and calculated corresponding ΔpNa (○). *F. succinogenes* S85 resting cells (10 mg of protein · ml^{-1}) washed in solutions containing 25 mM Na^+, 75 mM Na^+, and 100 mM, Na^+, were incubated in buffers containing 30 to 200 mM Na^+ in the presence of 1, 5, or 6 mM $Tm(DOTP)^{5-}$. Intracellular sodium concentrations were calculated from integrals measured in ^{23}Na NMR spectra (see Fig. 1).

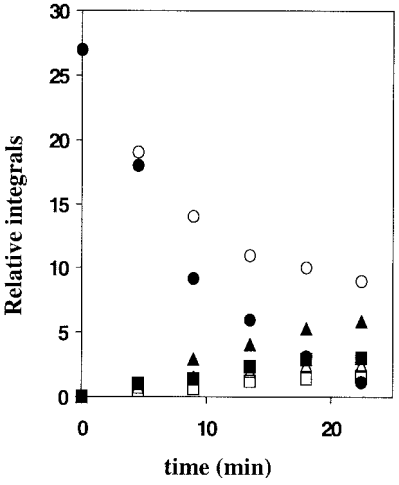

FIG. 3. In vivo ^{13}C NMR experiments. Time-dependent changes of ^{13}C NMR signal integrals of β and α [1-^{13}C]glucose (circles), [2-^{13}C]succinate (squares), and [1-^{13}C]glycogen (triangles) during 64 mM [1-^{13}C]glucose utilization by *F. succinogenes* S85 resting cells (10 mg of protein · ml^{-1}). Cells were washed in a buffer containing 75 mM Na^+ solution (solid symbols) or a buffer lacking sodium (open symbols) and then incubated in a buffer containing 75 mM Na^+.

tration (millimolar), and C_{ex} is the extracellular sodium concentration (millimolar).

The calculated sodium gradient (ΔpNa) was rather constant at about −55 mV for external sodium concentrations less than or equal to 100 mM. For higher extracellular [Na^+], the intracellular sodium concentration increased and ΔpNa decreased to −44 and −38 mV for 150 and 200 mM extracellular [Na^+], respectively.

The tight regulation of sodium gradients by *F. succinogenes* in the range of 30 to 100 mM Na^+ suggests that sodium ions play a very important role in its metabolism. In addition, we show that depletion of Na^+ ions during the washing steps led to irreversible damage to the cells that cannot be repaired by further addition of Na^+ (75 mM). The kinetics of [1-^{13}C]glucose utilization was monitored by in vivo ^{13}C NMR as previously described (11). The rate of glucose metabolism was decreased by a factor of 2 (Fig. 3) and the amount of glucose was finally degraded by a factor of 3 when the cells were prepared in the absence of sodium. Moreover, under these conditions the intracellular sodium concentration increased from 18 mM at 5 min to 54 mM at 30 min after the addition of sodium (75 mM), as shown by in vivo ^{23}Na NMR experiments, indicating that the cells were unable to maintain a stable Na^+ gradient (data not shown).

In this work we have shown that *F. succinogenes* is able to maintain a high transmembrane sodium gradient. In the range of 30 to 100-mM extracellular [Na^+]; ΔpNa was around −55 mV; it decreased slightly when the extracellular sodium concentration was increased. The intracellular free (visible) Na^+ concentrations, measured by ^{23}Na NMR in *Escherichia coli* (3, 12), in the halotolerant *Brevibacterium* sp. (12), or in the moderately halophilic *Vibrio costicola* (9), were similar to those measured in *F. succinogenes* for the same extracellular [Na^+]. As a result, the calculated ΔpNa values were comparable for these bacterial systems, although the ΔpNa value was slightly higher in the case of *E. coli* (−76 mV for 75 mM extracellular [Na^+]) (3). *E. coli* was shown to maintain a constant ΔpNa in the range of physiological extracellular sodium concentrations (4). The ΔpNa measured in *F. succinogenes* also appeared constant for concentrations of 30 to 100 mM extracellular Na^+, with the Na^+ concentration in the rumen ranging from 75 to 100 mM. Regulation of the intracellular sodium concentration is necessary, particularly in bacteria growing in a sodium-rich environment, in order to maintain an intracellular Na^+ concentration low enough not to interfere with enzyme activities, Na^+ often being inhibitory to these activities (15), and to utilize efficiently the ion gradient across the membrane for Na^+-driven systems (6). In particular, ΔpNa could be essential when ΔΨ is low, i.e., at more acidic pHs (13).

In this work we also showed that lack of sodium during cell-washing steps might lead to damage to cell membranes that cannot be reversed. A nonspecific effect on *F. succinogenes* cells of Na^+ as previously found on marine bacteria, such as osmotic effect or lysis prevention caused by interactions of the cations with the outer membrane (14, 15), might explain these results. This effect might also contribute to the sodium requirement for growth of *F. succinogenes*, as shown in some marine bacteria (16).

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