Generation of a Proton Motive Force by the Excretion of Metal-Phosphate in the Polyphosphate-accumulating Acinetobacter johnsonii Strain 210A∗

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The strictly aerobic, polyphosphate-accumulating Acinetobacter johnsonii strain 210A degrades its polyphosphate when oxidative phosphorylation is impaired. The endproducts of this degradation, divalent metal ions and inorganic phosphate, are excreted as a neutral metal-phosphate (MeHPO₄) chelate via the electrogenic MeHPO₄/H⁺ symport system of the organism. The coupled excretion of MeHPO₄ and H⁺ in A. johnsonii 210A can generate a proton motive force. In membrane vesicles and deenergized cells, a membrane potential of about -70 mV and transmembrane pH gradient of about -8 mV were formed in response to an imposed outwardly directed MeHPO₄ concentration gradient of 120 mM (initial value). The MeHPO₄ efflux-induced proton motive force could drive energy-requiring processes, such as the accumulation of L-proline and L-lysine and the synthesis of ATP via the membrane-bound F₄,F⁺, H⁺-ATPase. In vivo ³²P NMR studies of polyphosphate degradation in anaerobic cell suspensions revealed the presence of a considerable outwardly directed phosphate gradient across the cytoplasmic membrane corresponding to a MgHPO₄ concentration gradient of at least 100 mM. This MgHPO₄ concentration gradient was maintained for several hours. Thus, energy recycling by MeHPO₄/H⁺ efflux will contribute significantly to the overall production of metabolic energy from the degradation of polyphosphate in A. johnsonii 210A.

Activated sludge in wastewater treatment plants is enriched with polyphosphate-accumulating bacteria, e.g. from the strictly aerobic genus Acinetobacter, when alternating aerobic and anaerobic conditions are applied (Fuhs and Chen, 1975; Deinema et al., 1985). The polyphosphate metabolism of one of these strains, Acinetobacter johnsonii 210A, has been studied in detail (van Groenestijn et al., 1989a; 1989b; 1991). The subsequent conversion by adenylyl kinase of two molecules of ADP into one molecule of AMP and ATP enables A. johnsonii to regenerate AMP for the phosphotransferase reaction and may allow the organism to use its polyphosphate as a source of ATP when oxidative phosphorylation is impaired, e.g. under anaerobic conditions (van Groenestijn et al., 1988; van Groenestijn, 1988). During the degradation of metal-polyphosphate in A. johnsonii 210A, P₃ and metal divalent ions are excreted into the environment via the secondary MgHPO₄ transport system (van Groenestijn et al., 1988; 1991); van Veen et al., 1993b, 1994c).

Two enzymes are involved in the degradation of polyphosphate in A. johnsonii 210A: (i) polyphosphatase and (ii) polyphosphate:AMP phosphotransferase (van Groenestijn et al., 1989a). Polyphosphatase catalyzes the hydrolysis of polyphosphate to P₃ (Bonting et al., 1993b). The activity of this enzyme enables the organism to use metal-polyphosphate as a source of P₃ and divalent cations when the environmental concentrations of these nutrients are limiting (van Groenestijn and Deinema, 1985; van Groenestijn et al., 1988). Polyphosphate:AMP phosphotransferase catalyzes the phosphorylation of AMP to ADP with polyphosphate as phosphoryl donor (van Groenestijn et al., 1989a; Bonting et al., 1991). The subsequent conversion by adenylyl kinase of two molecules of ADP into one molecule of AMP and ATP enables A. johnsonii to regenerate AMP for the phosphotransferase reaction and may allow the organism to use its polyphosphate as a source of ATP when oxidative phosphorylation is impaired, e.g. under anaerobic conditions (van Groenestijn et al., 1987; van Groenestijn, 1988). During the degradation of metal-polyphosphate in A. johnsonii 210A, P₃ and metal divalent ions are excreted into the environment via the secondary MgHPO₄ transport system (van Groenestijn et al., 1988; van Veen et al., 1993b, 1994c).

Besides the direct synthesis of ATP via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway, A. johnsonii 210A may conserve metabolic energy from polyphosphate degradation by the reversed process of MeHPO₄ uptake via the secondary MeHPO₄ transport system (van Veen et al., 1993a, 1993b). During MeHPO₄ uptake, the energy of the electrochemical proton gradient is converted into the energy of a chemical MeHPO₄ gradient, whereas during MeHPO₄ efflux the energy of a chemical MeHPO₄ gradient may be converted back into the energy of an electrochemical proton gradient. In this paper, experimental support is given for this energy recycling mechanism. Energy transduction to electrogenic MeHPO₄/H⁺ efflux was studied in deenergized cells and mem-
brane vesicles of A. johnsonii 210A. In addition, in vivo $^{31}$P NMR was used to examine polyphosphate degradation and MeHPO$_4^-$ efflux in cells under physiological conditions.

**EXPERIMENTAL PROCEDURES**

**Cells Growth and Preparation of Membrane Vesicles—**A. johnsonii 210A was grown at 30°C in a Tris-buffered medium (pH 7.0) supplemented with 20 mM sodium butyrate and 5 mM or 20 mM sodium phosphate for cultivation of high and low P$_7$-grown cells, respectively (van Veen et al., 1993a). Cells were harvested by centrifugation (7,000 × g, 10 min). Membrane vesicles were prepared by osmotic lysis of high P$_7$-grown cells exposed to lithium chloride, a high concentration of lysozyme, and a temperature shock (van Veen et al., 1993b).

**Polyphosphate Degradation in Cells—**High P$_7$-grown cells containing polyphosphate granules and low P$_7$-grown control cells, in which polyphosphate granules were absent, were washed twice in ice-cold 50 mM Tris-HCl (pH 7.8). To permeabilize the outer membrane, cells were given an EDTA treatment as described previously (van Veen et al., 1993a). Subsequently, cells were washed in ice-cold 50 mM Tris-HCl (pH 7.8) and resuspended in this buffer to about 5.5 mg of protein/ml. The cell suspensions were transferred to Hungate tubes, flushed for 10 min with oxygen-free N$_2$, and incubated anaerobically at 30°C. Cell suspensions were transferred to Hungate tubes, flushed for 10 min with oxygen-free N$_2$, and incubated anaerobically at 30°C. TPP$^+$ accumulation was monitored. The induction of a proton motive force (Ap) by the oxidation of glucose under aerobic conditions was checked by measuring the endogenous respiration rate of the cells. Cells were centrifuged (7,000 × g, 10 min) and the supernatant was discarded. The buffer inside the vessel was flushed for 10 min with oxygen-free N$_2$, and the cell suspension was transferred to a TPP$^+$-selective electrode (Buffer A supplemented with 5 mM potassium phosphate (pH 7.5) and diluted into 10 mM potassium phosphate (pH 7.5), and diluted into 10 mM potassium phosphate (pH 7.5), and diluted into 10 mM potassium phosphate (pH 7.5)). 31P NMR Spectroscopy of Intact Cells—Low P$_7$-grown cells were washed and suspended to about 7 mg of protein/ml in 150 mM potassium phosphate (pH 7.0) supplemented with 5 mM MgSO$_4$. To allow the synthesis of polyphosphate, cells were kept under continuous aeration with oxygen-free N$_2$ for 60 min of incubation, cells were washed twice in 50 mM Tris-Cl (pH 7.0), and resuspended in 50 mM Tris-Cl (pH 7.8) supplemented with 10 mM potassium chloride and 2 mM MgSO$_4$. Subsequently, the cell suspension was kept in a 10-mm NMR tube and gassed with argon using an air-lift system. 31P NMR experiments were recorded using a 10-mm broadband probehead in a Bruker AMX500 spectrometer operating at 202.45 MHz for phosphorus. Spectra were acquired at 25°C, without proton decoupling, using a 45° flip angle and 5.8-s repetition delay in 16,000 data points. Phosphorus resonances were referenced with respect to external 85% H$_3$PO$_4$ as a standard.

**Determination of Membrane Potential—**The membrane potential ($\Delta\psi$, interior negative) in cells was determined from the distribution of the lipophilic tetraphenylphosphonium ion (TPP$^+$), using a TPP$^+$-selective electrode (Shibino et al., 1978). The membrane potential of the $\Delta\psi$ in cells during polyphosphate degradation, 800 µl of 50 mM Tris-HCl (pH 7.8) supplemented with 5 mM potassium cyanide and 5 µM TPP$^+$ was added to the TPP$^+$ electrode vessel. The vessel was sealed with a rubber septum. The buffer inside the vessel was flushed for 10 min with oxygen-free N$_2$. After 15 min, cell samples were injected into the vessel after which TPP$^+$ accumulation was monitored. The induction of a $\Delta\psi$ by MeHPO$_4^-$ efflux in energized cells was monitored with a TPP$^+$-electrode by diluting MeHPO$_4^-$-loaded cells 100-fold in 20 mM potassium Pipes (pH 7.5) containing 50 µM of chloromethyl phenylphosphonate (Buffer A). Cells were equilibrated overnight at 4°C in Buffer A supplemented with 5 mM potassium phosphate (pH 7.5) (about 3 mM MgHPO$_4^-$ (van Veen et al., 1994c)). The cell suspensions were concentrated to about 14 mg of protein/ml. An outwardly directed MeHPO$_4^-$ concentration gradient of about 120 mM was imposed by diluting preloaded cells 100-fold into 20 mM potassium Pipes (pH 7.5) containing 50 µM of chloromethyl phenylphosphonate (Buffer A). Deenergized cells equilibrated in Buffer A without added P$_7$ served as a control. Membrane vesicles in 10 mM potassium Pipes (pH 7.5) supplemented with 10 mM MgSO$_4$ (Buffer C) were equilibrated in the presence and absence of 5 mM potassium phosphate (pH 7.5), and diluted into 10 mM potassium Pipes (pH 7.5) (Buffer D) as described for deenergized cells.

**Determination of Membrane Potential—**The membrane potential ($\Delta\psi$, interior negative) in cells was determined from the distribution of the lipophilic tetraphenylphosphonium ion (TPP$^+$), using a TPP$^+$-selective electrode (Shibino et al., 1978). The membrane potential of the $\Delta\psi$ in cells during polyphosphate degradation, 800 µl of 50 mM Tris-HCl (pH 7.8) supplemented with 5 mM potassium cyanide and 5 µM TPP$^+$ was added to the TPP$^+$ electrode vessel. The vessel was sealed with a rubber septum. The buffer inside the vessel was flushed for 10 min with oxygen-free N$_2$. After 15 min, cell samples were injected into the vessel after which TPP$^+$ accumulation was monitored. The induction of a $\Delta\psi$ by MeHPO$_4^-$ efflux in energized cells was monitored with a TPP$^+$-electrode by diluting MeHPO$_4^-$-loaded cells 100-fold in Buffer B supplemented with 4 µM TPP$^+$. The ability of deenergized cell to generate a proton motive force ($\Delta\psi$) by the oxidation of glucose under aerobic conditions was checked by measuring the $\Delta\psi$ (interior negative) as described previously (van Veen et al., 1993a). For calculations, an internal cell volume of 3 µl/mg of protein was used (Kaback and Barnes, 1971; Bakker and Mangerich, 1981). Measurements were corrected for concentration-dependent nonspecific probe binding according to the model of Lokoma et al. (1982). For qualitative measurements of a MeHPO$_4^-$ efflux-induced $\Delta\psi$ in membrane vesicles, MeHPO$_4^-$-loaded membrane vesicles were diluted 100-fold in Buffer D supplemented with 9 µs of the membrane potential indicator 3,3'-di-propylthiacarbocyanine iodide (DiSC$_3$(5)). The $\Delta\psi$-dependent fluorescence was excited at 490 nm and emission was monitored at wavelengths of 637 and 667 nm, respectively, with slit widths of 10 nm. The fluorescence signal was averaged over time intervals of 0.3 s and calibrated in a $\Delta\psi$ range of −44 mV to −90 mV by measuring the fluorescence as a function of an artificially imposed potassium diffusion potential (van Veen et al., 1993b).

**Determination of Internal pH—**The internal pH in cells and membrane vesicles was estimated from the fluorescence of the pH indicator BCECF (van Veen et al., 1993c). Cells were preloaded with BCECF by an acid shock treatment as described by Molenaar et al. (1991), washed five times with 1 ml of Buffer A with or without 5 mM potassium phosphate, respectively, and resuspended in these buffers to about 5 mg of protein/ml. The cell suspensions were transferred to 10 mM potassium Pipes (pH 7.5) containing 50 µM of chloromethyl phenylphosphonate (Buffer A). Deenergized cells equilibrated in Buffer A without added P$_7$ served as a control. Membrane vesicles in 10 mM potassium Pipes (pH 7.5) supplemented with 10 mM MgSO$_4$ (Buffer C) were equilibrated in the presence and absence of 5 mM potassium phosphate (pH 7.5), and diluted into 10 mM potassium Pipes (pH 7.5) (Buffer D) as described for deenergized cells.

**Amino Acid Uptake Assays—**The uptake of 1.62 µM L-lysine and 1.55 µM L-proline in membrane vesicles was measured via the filtration method (Kaback, 1974) as described previously (van Veen et al., 1994a).

**Amino Acid Transport Assays—**The uptake of 1.62 µM L-lysine and 1.55 µM L-proline in membrane vesicles was measured via the filtration method (Kaback, 1974) as described previously (van Veen et al., 1994a).

**Energy Recycling by Polyphosphate Degradation—**The experiments shown in Fig. 1 demonstrate the ability of A. johnsonii 210A to conserve metabolic energy from the degradation of polyphosphate. In this study, high and low P$_7$-grown cells were used. High P$_7$-grown cells showed a relatively high phosphorus accumulation level of 4.1 µmol of phosphorus/mg of protein due to the presence of one or two large metal-polyphosphate granules in the cytoplasm. In contrast, low P$_7$-grown control cells were devoid of these granules and contained only 0.8 µmol of phosphorus/mg of protein. During aerobic incubation for 2 h in the absence of an exogenous carbon and energy source, high P$_7$-grown cells hardly excerted MeHPO$_4^-$ Their cellular ATP level and $\Delta\psi$ were similar to those observed in low P$_7$-grown cells. The aerobic incubation period was followed by an anaerobic one. Under the latter condition, high P$_7$-grown cells rapidly degraded polyphosphate, resulting in the excretion of MeHPO$_4^-$ at an initial rate of about 3 nmol/min/mg of protein (Fig. 1A). High P$_7$-grown cells were able to...
maintain a significant intracellular ATP concentration and $\Delta\psi$ for at least 8 h under these conditions, whereas in low Pi-grown cells the levels of these parameters strongly decreased within 1 h (Fig. 1, B and C).

**P$_i$ Gradient during Degradation of Polyphosphate—** Polyphosphate degradation was studied in *A. johnsonii* 210A using *in vivo* $^3$P NMR (Fig. 2). During the first 8 h of anaerobiosis about 65% of soluble polyphosphates was degraded (Fig. 2A). Strikingly, intracellular P$_i$ accumulated up to 150 mm in the course of polyphosphate degradation (Fig. 2B). Since the external P$_i$ concentration remained below 11 mm, an outwardly directed P$_i$ gradient ($Z\Delta\rho_{P_i}$) of 100–160 mV was maintained for 4 h (Fig. 2C). In view of (i) the important role of polyphosphate in the production of metabolic energy during anaerobiosis, (ii) the large outwardly directed P$_i$ gradient that is maintained during polyphosphate degradation, and (iii) the presence of a phosphate carrier that mediates the translocation of MeHPO$_4^-$ via an electrogenic H$^+$ symport mechanism, it was of interest to study energy transduction coupled to MeHPO$_4^-$ efflux in *A. johnsonii* 210A.

**MeHPO$_4^-$ Efflux in Deenergized Cells—** In order to study the recycling of metabolic energy by MeHPO$_4^-/H^+$ efflux in the absence of polyphosphate metabolism, cells of *A. johnsonii* 210A were depleted of polyphosphate and other endogenous energy reserves by aerobic incubation in the presence of the uncoupler a-dinitrophenol. As was shown previously (van Veen et al., 1993a), the cells retain the secondary MeHPO$_4^-$ transport system in an active form during the deenergization procedure and remain readily energizable after removal of a-dinitrophenol.

In deenergized cells, MeHPO$_4^-/H^+$ efflux was coupled to the generation of a $\Delta\rho$. Thus, the lipophilic cation TPP$^+$ was accumulated almost 15-fold when an outwardly directed MeHPO$_4^-$ gradient (initial value of $-120$ mV) was imposed artificially by dilution of deenergized, MeHPO$_4^-$-loaded cells into MeHPO$_4^-$-free buffer (Fig. 3A). The maximum TPP$^+$ accumulation level suggested the generation of a $\Delta\psi$ of about $-70$ mV. No significant accumulation of TPP$^+$ was observed in unloaded cells or in loaded cells in which the $\Delta\rho$ was dissipated by valinomycin plus nigericin (each 2 nmol/mg of protein) (Fig. 3A). To monitor the changes in the intracellular pH by MeHPO$_4^-/H^+$ efflux, deenergized cells were loaded with the fluorescent pH indicator BCECF. A rapid alkalinization of the internal milieu was observed when an outwardly directed MeHPO$_4^-$ gradient (initial value of 120 mV) was imposed (Fig. 3B). The addition of valinomycin plus nigericin (each 2 nmol/mg of protein) resulted in the decrease of the internal pH to base-line levels observed in unloaded cells. With a constant external pH during MeHPO$_4^-$ efflux, the degree of alkalinization of the cytoplasmic pH equalled a transmembrane pH gradient ($-Z\Delta\rho_{H^+}$) of about $-7$ mV under these conditions.

The $\Delta\rho$ induced by MeHPO$_4^-/H^+$ efflux in deenergized cells could drive the synthesis of ATP from endogenous ADP and P$_i$ via the membrane-bound H$^+$-ATPase (Fig. 4). ATP levels remained very low in control cells in which a MeHPO$_4^-$ gradient was absent, e.g. through dilution of MeHPO$_4^-$-loaded cells into buffers containing MeHPO$_4^-$, at a concentration equivalent to the internal one, or of unloaded cells into MeHPO$_4^-$-free buffer. A significant synthesis of ATP was observed when MeHPO$_4^-$-loaded cells were diluted 100-fold into MeHPO$_4^-$-free buffer. This synthesis is transient due to the rapid decrease of the MeHPO$_4^-$ gradient.

**MeHPO$_4^-$ Efflux in Membrane Vesicles—** In membrane vesicles, the mechanism of energy coupling to secondary transport of solutes can be studied in the absence of their metabolism by cytoplasmic enzymes. Membrane vesicles therefore offer an excellent model system to study energy transduction coupled to MeHPO$_4^-/H^+$ efflux in *A. johnsonii* 210A. The generation of a MeHPO$_4^-$ efflux-induced $\Delta\rho$ was monitored in membrane vesicles using the fluorescent $\Delta\rho$-indicator DiSC$_{3}(5)$ (Fig. 5A). Upon imposition of an outwardly directed MeHPO$_4^-$ gradient (initial value of 120 mV) in membrane vesicles, a rapid fluorescence quenching of DiSC$_{3}(5)$ was observed corresponding to a $\Delta\rho$ of about $-63$ mV (Fig. 5A). The quenched fluorescence signal was elevated to the baseline level observed in unloaded
membrane vesicles after dissipation of the Δp by valinomycin plus nigericin (each 1 nmol/mg of protein). The formation of a ΔpH by MeHPO₄/H⁺ efflux was demonstrated by continuous recording of the fluorescence intensity of BCECF trapped within the membrane vesicles (Fig. 5B). Imposition of an outwardly directed MeHPO₄ gradient (initial value of 120 mV) resulted in an alkalinization of the intravesicular pH and the MeHPO₄/H⁺ efflux in membrane vesicles loaded cells in which an outwardly directed MeHPO₄ gradient (initial value of 120 mV) was imposed artificially (C).

Membrane vesicles of this organism contain several cation–amino acid transport systems that couple amino acid translocation to the Δp (van Veen et al., 1994a). Significant levels of L-lysine and L-proline accumulation were observed in MeHPO₄-loaded membrane vesicles that were diluted 100-fold in Me-

FIG. 4. Effect of a MeHPO₄ efflux-induced Δp on the synthesis of ATP in deenergized cells of A. johnsonii 210A. ATP concentrations were determined in MeHPO₄-loaded (○) and unloaded cells (●) in the absence of a MeHPO₄ concentration gradient, and in MeHPO₄-loaded cells in which an outwardly directed MeHPO₄ gradient (initial value of 120 mV) was imposed artificially (●).

FIG. 5. Generation of a Δψ (panel A) and ΔpH (panel B) by MeHPO₄/H⁺ efflux in membrane vesicles of A. johnsonii 210A. Measurements were performed in MeHPO₄-loaded membrane vesicles in the presence of an outwardly directed MeHPO₄ gradient (initial value of 120 mV) (trace I) and in unloaded membrane vesicles (trace II). The traces are offset for the purpose of display. A, trace I received 1 nmol each of valinomycin and nigericin/mg of protein at the time indicated by the second arrow; trace II received no addition. B, trace I represents two independent trials that received no addition or 1 nmol each of valinomycin and nigericin/mg of protein at the time indicated by the arrow; trace II received no addition.

HPO₄⁻-free buffer (Fig. 6). Accumulation of amino acids was not observed (i) upon dissipation of the MeHPO₄ efflux-induced Δp by valinomycin plus nigericin (each 1 nmol/mg of protein) and (ii) in the absence of an outwardly directed MeHPO₄ gradient, e.g. when MeHPO₄-loaded vesicles were diluted into buffers containing MeHPO₄ at a concentration equimolar to the internal one or when unloaded membrane vesicles were diluted into MeHPO₄-free buffer. These experiments show that a MeHPO₄ efflux-induced Δp can drive the uptake and accumulation of solutes in A. johnsonii 210A.

DISCUSSION

A. johnsonii 210A is a strictly aerobic nonfermentative bacterium. When oxidative phosphorylation is impaired, it degrades the metal-polyphosphate that was accumulated under aerobic conditions. The results of this investigation demonstrate that during this degradation, the organism is able to maintain its Δp and intracellular ATP at levels comparable with those observed under aerobic conditions. Two mechanisms for the conservation of metabolizable energy from polyphosphate degradation have been suggested in A. johnsonii 210A: (i) the direct synthesis of ATP from polyphosphate via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway (van Groenestijn et al., 1987; Van Groenestijn, 1988) and (ii) the generation of a Δp by the excretion of MeHPO₄, a major end-product of metal-polyphosphate degradation, together with H⁺ (van Veen et al., 1993a, 1993b).

Recently, the energetics and mechanism of the secondary MeHPO₄ transport system of A. johnsonii 210A were examined in detail. The MeHPO₄ carrier catalyzes the coupled movement of a neutral MeHPO₄ chelate and a proton via an electrogenic symport mechanism (van Veen et al., 1993a, 1994c). Thus, the driving force for MeHPO₄ translocation via this transport system is the sum of forces supplied by the Δp (Δψ = ΔpH) and the MeHPO₄ concentration gradient (ΔpMeHPO₄): Δp = 2ΔpMeHPO₄. A steady state is reached when Δp = −2ΔpMeHPO₄. During MeHPO₄ uptake, the Δp will exceed the −2ΔpMeHPO₄, whereas MeHPO₄ efflux occurs when the −2ΔpMeHPO₄ exceeds the Δp. In previous work, the generation of a Δp by electroneutral MeHPO₄/H⁺ efflux was indirectly indicated by (i) the stimulation of MeHPO₄ efflux from cells by the uncoupler
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**Fig. 7. Schematic presentation of the coupling of MeHPO₄⁻/H⁺ efflux to different energy requiring processes in A. johnsonii 210A.**

1. H⁺-ATPase; 2. secondary MeHPO₄⁻/H⁺ carrier; 3. lysine/proton symporter; 4. sodium/proton antiporter; 5. proline/sodium symporter. Cytoplasmic membrane, -lysine, -proline, protons, sodium ions, and H⁺/ATP and H⁺/Na⁺ stoichiometries are indicated by CM, LYS, PRO, H⁺, Na⁺, a, and m, respectively.

α-dinitrophenol and by N,N'-dicyclohexylcarbodiimide, an inhibitor of the F,F⁻, H⁺-ATPase (van Groenestijn, 1988), (ii) the enhancement by protonophore carbonyl cyanide(3-chlorophenyl)hydrazone of MeHPO₄⁻ efflux from proteoliposomes containing reconstituted MeHPO₄⁻ carrier protein (van Veen et al., 1993b), and (iii) the retardation of MeHPO₄⁻ efflux from proteoliposomes by an artificially imposed ΔpH and/or Δψ (van Veen et al., 1993b).

The results presented in this study further corroborate the generation of a Δψ by MeHPO₄⁻/H⁺ efflux. Thus, the generation of a Δψ by the efflux of MeHPO₄⁻ was demonstrated directly by the fluorescence quenching of the Δψ probe DiSC₃(5) in membrane vesicles and by the accumulation of TPP⁺ in deenergized cells under these conditions. In both systems, imposition of an outwardly directed MeHPO₄⁻ gradient of 120 mV (initial value) resulted in the generation of a Δψ of about −60 to −70 mV. The generation of a transmembrane pH gradient by MeHPO₄⁻/H⁺ efflux was shown by the fluorescence enhancement of the pH-probe BCECF, which was entrapped in the lumen of the membrane vesicles and deenergized cells. With the external pH remaining fairly constant during MeHPO₄⁻ efflux, a −2ΔpH of about −8 mV was built up upon imposition of a MeHPO₄⁻ gradient of 120 mV (initial value).

The MeHPO₄⁻ efflux-induced Δψ could be coupled to different metabolic energy requiring processes as summarized in Fig. 7. In membrane vesicles of A. johnsonii 210A, MeHPO₄⁻/H⁺ efflux could drive the accumulation of L-proline and L-lysine. Recent transport studies in membrane vesicles of this organism revealed the presence of single, high-affinity lysine and proline carriers that mediate the electrogenic symport of lysine and a proton and of proline and a sodium ion, respectively (van Veen et al., 1994a). The Na⁺ contamination in the uptake buffers (up to 150 mM) greatly exceeds the low Kₑₐ of the proline carrier for Na⁺ (Kₑₐ = 26 μM Na⁺). Na⁺ is therefore present in sufficient amounts to allow Na⁺/proline symport. In addition, A. johnsonii 210A possesses a Na⁺/H⁺ antipporter, which converts the Δψ into a sodium motive force (van Veen et al., 1994a). In view of the driving forces for lysine and proline uptake, 2Δψ − ZΔpH and Δψ − ZΔpNa, respectively, the almost 2-fold higher accumulation level of lysine compared with that of proline points to an effective generation of a Δψ by MeHPO₄⁻/H⁺ efflux under the experimental conditions. This conclusion is consistent with the direct measurements of the composition and magnitude of the MeHPO₄⁻ efflux-induced Δψ in membrane vesicles and deenergized cells. Besides solute accumulation, the MeHPO₄⁻ efflux-induced Δψ could drive the synthesis of ATP via the membrane-bound H⁺-ATPase in deenergized cells of A. johnsonii 210A. The results from these in vitro studies demonstrate the potential of MeHPO₄⁻/H⁺ efflux as an energy recycling mechanism in A. johnsonii 210A (Fig. 7).

³¹P NMR was used for in vivo studies of Pᵢ gradients formed by the degradation of polyphosphate in anaerobic cell suspensions of A. johnsonii 210A. The concentration variations in internal Pᵢ were much greater in magnitude and range than those in the external medium. The cells were able to maintain an outwardly directed Pᵢ gradient of 100–160 mM for 4 h. Due to the high internal concentrations of Mg²⁺ (up to 40 mM (Bonting et al., 1993a)) and Pᵢ (up to 150 mM) during the degradation of magnesium-polyphosphate, the intracellular MgHPO₄ concentration will have reached saturating levels of about 20–30 mM (van Veen et al., 1994c). Thus, a substantial outwardly directed MgHPO₄ concentration gradient (of at least 100 mM) was present during the first 3 h of polyphosphate degradation, allowing MeHPO₄⁻/H⁺ efflux to be an effective energy conserving mechanism.

Under conditions of polyphosphate synthesis, the MeHPO₄⁻ carrier and the ATP and binding protein-dependent Pᵢ uptake system of A. johnsonii 210A enable the organism to efficiently acquire Pᵢ from its habitat through uptake of the predominant Pᵢ species (van Veen et al., 1994c). However, the latter transport system has to be inactivated during the degradation of polyphosphate to prevent the reaccumulation of Pᵢ with a concomitant waste of ATP. The inactivation of the primary Pᵢ uptake system of A. johnsonii 210A may be exerted through trans-inhibition by the high internal Pᵢ concentration that is established during the degradation of polyphosphate. Trans-inhibition by Pᵢ has been described for a number of phosphate bond energy-driven uptake systems, including the Pᵢ transporter of Lactococcus lactis (Poolman et al., 1987), and the sn-glycerol-3-P permease (Ugp) and phosphate-specific transport system (Pet) of Escherichia coli (Medveczky and Rosenberg, 1971; Brzoska et al., 1994).

Fermentative bacteria continuously excrete relatively large quantities of lactic acid and other organic acids into the environment. In their "energy recycling model," Michels et al. (1979) proposed that electrogenic efflux of these organic end-products via ion symport systems may lead to the generation of an electrochemical ion gradient across the cytoplasmic membrane, thus providing metabolic energy to the cell. In recent years evidence has been obtained for H⁺-linked carrier-mediated excretion of lactate in lactic acid bacteria (Otto et al., 1980, 1982; Simpson et al., 1983a, 1983b) and enteric bacteria (ten Brink and Konings, 1980), and Na⁺-linked excretion of succinate in Selenomonas ruminantium (Michel and Macy, 1990). Other organic acids, like acetate, may also be excreted via carrier-mediated processes (Driessen and Konings, 1990). The observations in A. johnsonii 210A (this work) extend the energy recycling model to the excretion of inorganic endproducts.

Thus, the results show that a Δψ is generated by the electrogenic excretion of MeHPO₄⁻ and H⁺ via the secondary MeHPO₄- transport system of this organism. In view of the evidence for cotransport of Pᵢ and divalent cations in other biological systems (van Veen et al., 1994b, 1994d), energy recycling by the excretion of metal-phosphate chelates may be a more general mechanism for the conservation of metabolic energy in polyphosphate-accumulating microorganisms. Recycling of metabolic energy by the excretion of inorganic endproducts may also have interesting implications for the efflux of NH₄⁺ by Ureaplasma species (Masover et al., 1977; Smith et al., 1993) and the excretion of sulfate by Thiobacillus species and other sulfur-oxidizing bacteria (Kelly, 1988).
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In conclusion, A. johnsonii 210A is able to use metal-polyphosphate as a source of metabolic energy during anaerobiosis by (i) the direct synthesis of ATP via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway and (ii) the generation of a Δp via the coupled excretion of MeHPO₄⁻ and H⁺. Polyphosphatase may enhance the latter energy recycling mechanism by providing the MeHPO₄⁻ efflux process with a continuous supply of Pᵢ and divalent metal ions. As a consequence of energy recycling by MeHPO₄⁻ excretion, less ATP has to be hydrolyzed via the H⁺-ATPase to generate a Δp when oxidative phosphorylation is impaired. Conservation of metabolic energy from metal-polyphosphate degradation may enable A. johnsonii 210A to survive alternating aerobic/anaerobic conditions as encountered in certain natural habitats and in wastewater treatment plants.

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