A Na\textsuperscript{+}-translocating Pyrophosphatase in the Acetogenic Bacterium *Acetobacterium woodii*

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The anaerobic acetogenic bacterium *Acetobacterium woodii* employs a novel type of Na\textsuperscript{+}-motive anaerobic respiration, caffeate respiration. However, this respiration is at the thermodynamic limit of energy conservation, and even worse, in the first step, caffeate is activated by caffeyl-CoA synthetase, which hydrolyzes ATP to AMP and pyrophosphate. Here, we have addressed whether or not the energy stored in the anhydride bond of pyrophosphate is conserved by *A. woodii*. Inverted membrane vesicles of *A. woodii* have a membrane-bound pyrophosphatase that catalyzes pyrophosphate hydrolysis at a rate of 70–120 milliunits/mg of protein. Pyro-driven pyrophosphatase that catalyzes pyrophosphate is the second primary Na\textsuperscript{+}K\textsubscript{m}a

EXPERIMENTAL PROCEDURES

**Growth of Cells and Preparation of Vesicles—*A. woodii* (DSM 1030) was grown under anaerobic conditions using 20 mm fructose as substrate as described (5, 7). The preparation of vesicles was done as described but slightly modified. For preparation of vesicles, the growth medium was supplemented with 420 mm sucrose and 8.1 mm MgSO\textsubscript{4}. 5 liters of medium were inoculated (4%), and the absorbance was followed at 600 nm. At *A*\textsubscript{600} = 0.7–0.9, 70 μg of penicillin G/ml were added to the medium to induce protoplast formation. During further incubation, *A. woodii* formed protoplasts as monitored by microscopic observations. After 20 h, the culture consisted almost entirely of spherical forms that were highly sensitive to low osmolarity. These protoplasts were harvested aerobically by centrifugation (6250 × g, 20 min, 4 °C) and washed in 25 mM Pipes (sodium-free)/KOH buffer (pH 6.8) containing 25 mM MgSO\textsubscript{4} and 420 mM sucrose. After washing the protoplasts, they were resuspended in a total volume of 300 ml and incubated with lysozyme (1 mg/ml) for 30 min at room temperature. The protoplasts were centrifuged (6250 × g, 20 min, 4 °C) and resuspended in 25 mm Pipes (sodium-free)/KOH buffer (pH 6.8) containing 25 mm MgSO\textsubscript{4} and 420 mm sucrose. After washing the protoplasts, they were resuspended in a total volume of 300 ml and incubated with lysozyme (1 mg/ml) for 30 min at room temperature. The protoplasts were centrifuged (6250 × g, 20 min, 4 °C) and resuspended in 25 mm Pipes/KOH buffer (pH 6.8) containing 25 mm MgSO\textsubscript{4} and 420 mm sucrose. These protoplasts were passed through a French pressure cell at 41 megapascals and centrifuged three times (4500 × g, 35 min, 4 °C). The resulting supernatant (i.e. crude vesicles) was centrifuged further by ultracentrifugation (120,000 × g, 40 min, 4 °C). The pellet was washed in Pipes/KOH buffer (pH 6.8) containing 25 mm MgSO\textsubscript{4} and 420 mm sucrose and centrifuged again. The resulting pellet was resus-
Pended in the same buffer in a volume of 3 ml. Protein concentrations were determined by the method of Bradford (16).

**PPi Hydrolysis**—Pyrophosphatase (PPase) activity was determined by measuring the released phosphate using a method described by Heinonen and Lathi (17).

**Measurement of Na\(^+\) Translocation**—The experiments were performed under aerobic conditions in Pipes/KOH buffer (pH 6.8) containing 25 mM MgSO\(_4\), 420 mM sucrose, and 1.9 mM NaCl as described (7). The sodium and protein concentrations used are indicated. In a 1.5-ml Eppendorf cup, the vesicles, buffer, supplements (17 μM valinomycin, 1 mM N,N',N'-dicyclohexylcarbodiimide, NaCl, and 150 mM KCl), and \(^{22}\)NaCl (final activity of 0.5 μCi/ml) were combined and incubated at 30 °C for 120 min to assure equilibration of \(^{22}\)Na\(^+\) before the reaction was started with potassium pyrophosphate (final concentration of 1 mM or as indicated). 100 μM N,N,N',N'-tetracyclohexyl-1,2-phenylenedioxycarbamidem (ETH 2120) and 3,5-di-tert-butylhydroxybenzylidenemalonitrile (SF 6847) were added 6 min before the addition of PPi. 100-μl samples were withdrawn from the cup and passed over a column (0.5 × 3.2 cm) of Dowex 50-WX8 (100–200-mesh) (7). The vesicles were collected by washing the column with 1 ml of 420 mM sucrose. The radioactivity in the eluate was determined by liquid scintillation counting. For elucidating the effect of Mg\(^2+\) on the transport activity, the vesicles were prepared in the same buffer except that MgSO\(_4\) was omitted. MgSO\(_4\) and EDTA were added from stock solutions before the addition of \(^{22}\)NaCl.

**Determination of Na\(^+\) Concentration**—The Na\(^+\) concentration in the buffer was determined with an Orion 84-11 ROSS sodium electrode (Thermo Electron Corp., Witchford, UK) as described (18).

**RESULTS**

Inverted membrane vesicles (IMVs) of *A. woodii* prepared in Pipes/KOH buffer (pH 6.8) that also contained 25 mM MgSO\(_4\), 420 mM sucrose, and 1.9 mM NaCl were incubated at 30 °C. Upon the addition of PPi, it was hydrolyzed with a rate of 70–120 milliunits/mg (1 unit corresponding to 1 μmol of PPi/min at 1.9 mM NaCl). PPi hydrolysis was dependent on the external Na\(^+\) concentration. When no sodium was added to the assay, an activity of 68–74 milliunits/mg was measured (resulting from residual Na\(^+\) in the buffer and vesicles), but increasing Na\(^+\) concentrations led to increasing activities in a Michaelis-Menten-type fashion (Fig. 1A). The double-reciprocal plot is shown in Fig. 1B, and a \(K_m\) of 1.1 ± 0.4 mM and a \(V_{max}\) of 170 ± 12 milliunits/mg of protein were obtained. KCl and LiCl had only a marginal stimulatory effect. Pyrophosphate hydrolysis was dependent on the pyrophosphate concentration. Highest activities were obtained at 1 mM PPi (Fig. 2A). Pyrophosphate hydrolysis was accompanied by \(^{22}\)Na\(^+\) transport into the vesicles (Fig. 2B). As expected, \(^{22}\)Na\(^+\) transport increased with the pyrophosphate concentration. Also, at...
lower PPᵢ concentrations, the Na⁺ gradient established collapsed after some time, which may be due to PPᵢ depletion. There was a negligible activity at the lowest Na⁺ concentration achieved in the assay (900 μM, taking into account the buffer, vesicles, and potassium pyrophosphate), but transport activity increased with increasing Na⁺ concentration in a Michaelis-Menten fashion (Fig. 3A). The corresponding Lineweaver-Burk plot is shown in Fig. 3B. The Kₘ for Na⁺ transport was determined to be 7.2 ± 1.4 mM, and the Vₘₐₓ was 3.6 ± 0.25 nmol/min/mg of protein. This gives a Na⁺/PPᵢ ratio of 0.02.

The intravesicular Na⁺ concentration in the IMVs (and thus the magnitude of the established Na⁺ gradient) was also dependent on the concentration of Na⁺. At 0.9 mM NaCl, a 10-fold accumulation was observed. Increasing Na⁺ concentrations led to decreasing Na⁺ gradients. At 1.9 mM NaCl, the accumulation factor was 9-fold, and at 40.9 mM NaCl, it was only 2.2-fold. Because the accumulation factor was higher at lower sodium concentrations, all subsequent experiments were performed in the presence of 1.9 mM NaCl.

It has been described previously that the PPhase activity in Rhodospirillum rubrum and the activity of PPhase isolated from mung beans are Mg²⁺−dependent (19, 20). To test the effect of Mg²⁺ on the PP₁-dependent Na⁺ transport at IMVs of A. woodii, IMVs had to be prepared in Mg²⁺−free buffer. Unfortunately, when IMVs were prepared in the absence of MgSO₄, the yield was very low, and the activity was very low, indicating that MgSO₄ stabilizes the IMVs. When these IMVs were added to Mg²⁺−free assay buffer, Na⁺ transport (at 1.9 mM NaCl) was very low, with 0.004 nmol/min/mg of protein. The addition of Mg²⁺ to a final concentration of 25 mM stimulated Na⁺ transport by 2000% to 0.08 nmol/min/mg of protein. When the Mg²⁺−containing assay was supplemented with EDTA, Na⁺ transport dropped to 0.01 nmol/min/mg of protein. These data show that PPhase-driven Na⁺ transport requires Mg²⁺.

If transport of ²²Na⁺ is electrogenic, a membrane potential should be established in turn should slow down ²²Na⁺ transport. To test this, we used valinomycin in combination with KCl to dissipate the potential (∆Ψ). KCl itself (0, 50, 100, and 150 mM) had no effect on Na⁺ transport. However, in combination with valinomycin, ²²Na⁺ transport was stimulated slightly (Fig. 4). This experiment indicates that PP₁-driven ²²Na⁺ transport is electrogenic.

PP₁-dependent Na⁺ transport should lead to the generation of a sodium ion potential that then could be used by the Na⁺/F₁F₀-ATP synthase of A. woodii to synthesize ATP. If this is true, inhibition of the Na⁺/F₁F₀-ATP synthase should lead to higher levels of ²²Na⁺ in the lumen of the vesicles. This was indeed observed. In the presence of the F₀-directed inhibitor N,N'-dicyclohexylcarbodiimide (1 mM, no extra sodium added to the assay), PP₁-driven accumulation of ²²Na⁺ was increased by 25%. However, the Na⁺ transport rate was not affected.

Pyrophosphatase activity could be directly or indirectly (via a primary H⁺/Na⁺ antiporter) coupled to Na⁺ transport. To discriminate between these possibilities, we used protonophores or sodium ionophores and analyzed their effect on ²²Na⁺ transport. The protonophore SF 6847 (100 μM) did not inhibit ²²Na⁺ accumulation (Fig. 5). The effectiveness of the uncoupler was ensured by its ability to dissipate an artificial ∆pH created by a NH₄⁺ diffusion potential (21). The sodium ionophore ETH 2120 (100 μM) completely inhibited ²²Na⁺ accumulation; moreover, a previously established Na⁺ gradient was dissipated immediately upon the addition of ETH 2120 (Fig. 5). These
data demonstrate that $^{22}$Na$^+$ transport is directly linked to pyrophosphatase activity.

**DISCUSSION**

Pyrophosphatases are commonly found enzymes. Next to soluble pyrophosphatases, membrane-bound enzymes have been found in bacteria, archaea, and plants (22–25). It has been shown that membrane-bound PPases couple PP$\text{I}$ hydrolysis with proton or sodium ion transport (23, 26–29). Proton-pumping PPases acidify vacuoles in plants (22). In bacteria, they have been proposed to support energy conservation under conditions of energy deprivation (30, 31), and a light-induced maintenance of PP$\text{I}$ levels by PPase has been suggested based on experiments with *Rhodobacter capsulatus* (32). PP$\text{I}$ is a by-product of many biosynthetic processes for macromolecules, such as RNA and DNA synthesis, polysaccharide synthesis, formation of fatty acyl-CoA, and amino acid activation, and is formed in several reactions for substrate activation by anaerobic bacteria, e.g. activation of sulfate by sulfate-reducing bacteria or in activation of benzoate derivatives during anaerobic degradation of these compounds (23, 33).

Here, we have demonstrated a Na$^+$-pumping pyrophosphatase at IMVs of *A. woodii*. The coupling efficiency was low, indicating leakiness of the vesicle system. Additionally, a second non-Na$^+$-motive pyrophosphatase may contribute to PP$\text{I}$, hydrolysis activity. This enzyme may be membrane-attached and thus also explain the different $K_m$ values for $^{22}$Na$^+$ transport and PP$\text{I}$ hydrolysis.

The overall ATP gain of caffeate respiration, as it is understood now, is very low. Electron flow from ferredoxin ($\sim 500$ to $\sim 420$ mV) to NAD$^+$ ($\sim 320$ mV) is accompanied with a free energy change of only $\sim 35$ to $\sim 19$ kJ/mol, and therefore, the Fno/Rnf complex allows for the synthesis of about one-third to one-half of an ATP. Thus, 2–3 mol of hydrogen have to be oxidized to get 1 ATP. Therefore, its highly economic for the cells to save the energy stored in PP$\text{I}$. The standard free energy stored in the PP$\text{I}$ bond is equivalent to $\sim 22$ kJ/mol. Assuming a membrane potential of $\sim 200$ mV, this would allow for translocation of 1 Na$^+$. Consequently, 3 mol of PP$\text{I}$, have to be hydrolyzed for the production of 1 mol of ATP. This is only a small amount of energy saved, but rationalizing that caffeate respiration is at the thermodynamic limit, this adds another piece to the overall energy balance. Additionally, energy may be conserved by electron bifurcation from NADH to ferredoxin in the process of caffeyl-CoA reduction and the subsequent Fno/Rnf complex-catalyzed ferredoxin oxidation (34, 15). Furthermore, in the steady state of caffeate respiration, the activation of caffeate is likely to be catalyzed by an energy-saving CoA loop from hydrocaffeyl-CoA to caffeate (35).

In summary, caffeate respiration involves an initial activation of the caffeate prior to its reduction by a AMP and PP$\text{I}$, forming caffeyl-CoA synthetase, yielding caffeyl-CoA. The produced PP$\text{I}$, as shown here, can be used by a membrane-bound pyrophosphatase to translocate Na$^+$ across the cytoplasmic membrane in *A. woodii*. Therefore, the energy stored in the PP$\text{I}$ bond is not lost but reinvested and conserved in a chemiosmotic Na$^+$ potential (Fig. 6).

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**FIGURE 6. Model of caffeate respiration in *A. woodii*.** Flow of electrons from electron donors (fructose or hydrogen) to the acceptor caffeate is shown. The PP$\text{I}$ generated by the caffeyl-CoA synthetase is used by the PPase to pump Na$^+$ across the membrane.
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