Sodium Dependence of Acetate Formation by the Acetogenic Bacterium Acetobacterium woodii

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Growth of Acetobacterium woodii on fructose was stimulated by Na⁺; this stimulation was paralleled by a shift of the acetate-fructose ratio from 2.1 to 2.7. Growth on H₂-CO₂ or on methanol plus CO₂ was strictly dependent on the presence of sodium ions in the medium. Acetate formation from formaldehyde plus H₂-CO₂ by resting cells required Na⁺, but from methanol plus H₂-CO₂ did not. This is analogous to H₂-CO₂ reduction to methane by Methanosarcina barkeri, which involves a sodium pump (V. Müller, C. Winner, and G. Gottschalk, Eur. J. Biochem. 178:519–525, 1988). This suggests that the reduction of methylene-tetrahydrofolate to methyltetrahydrofolate is the Na⁺-requiring reaction. A sodium gradient (Na⁺out/Na⁺in = 32, ΔpNa = −91 mV) was built up when resting cells of A. woodii were incubated under H₂-CO₂. Acetogenesis was inhibited when the ΔpNa was dissipated by monensin.

Many acetogenic and methanogenic bacteria grow and hence are able to gain energy by the reduction of CO₂ with H₂ to acetate and methane, respectively. Methanogens require sodium ions for growth and methane formation (22), and evidence has been provided that methylene-tetrahydrodromethanopterin reduction is coupled to the extrusion of Na⁺ (20). Thus, a primary sodium pump is operative, and the transmembrane electrochemical sodium gradient established can be utilized by the organisms for their energy expenditures, e.g., for solute transport (13), pH regulation under acidic conditions (24), or ATP synthesis via a secondary proton gradient (20). The energy metabolism of acetogenic bacteria fermenting H₂ plus CO₂ to acetate is still obscure. As is apparent from Fig. 1, 1 ATP is converted to ADP + Pᵢ in the formyl-tetrahydrofolate synthetase reaction, and 1 ATP is formed in the final step of acetogenesis. The ATP balance from substrate level phosphorylation is therefore zero. Clearly, additional mechanisms for energy conservation must be present. In this context, the discovery of an Na⁺/H⁺ antiporter in Clostridium thermocaccum by Terraciano et al. (26) was important. Because of this and the analogy of the two methylene-group reduction reactions methylene tetrahydrodromethanopterin + H₂ → methylene tetrahydrodromethanopterin (ΔG°′ = −20 kJ/reaction [15]) and methylene tetrahydrofolate + H₂ → methylene tetrahydrofolate (ΔG°′ = −57.3 kJ/reaction [8, 27]), it was hypothesized than an Na⁺ gradient would be also generated in acetogens (10). Such a gradient would allow these organisms to produce additional ATP during acetogenesis from H₂ plus CO₂. As a typical acetogen, we studied Acetobacterium woodii (1), and we show here that it requires Na⁺ for acetogenesis and that it generates an Na⁺ gradient across the cytoplasmic membrane.

(Preliminary results of this study were presented at the Annual Meeting of the Vereinigung für Allgemeine and Angewandte Mikrobiologie [R. Heise, V. Müller, and G. Gottschalk, Forum Mikrobiol. 12:54, 1989].)

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MATERIALS AND METHODS

Strains and growth of organisms. Acetobacterium woodii (DSM 1030) was obtained from the German Collection of Microorganisms (DSM), Braunschweig, Federal Republic of Germany. The medium was prepared according to the anaerobic techniques of Hungate (12) as modified by Bryant (4). The medium contained (in grams per liter): KH₂PO₄, 0.2; NH₄Cl, 0.25; NaCl, 1.16; MgSO₄ . 7H₂O, 1.45; CaCl₂, 0.11; KCl, 0.50; yeast extract, 2.0; plus trace element solution SL 9 and selenite-tungstate solution (28), 1 ml/liter each; and vitamin solution DSM 141, 20 ml/liter. The vitamin solution contained (in milligrams per liter): biotin, 2.0; folic acid, 2.0; pyridoxine hydrochloride, 10.0; thiamine hydrochloride, 5.0; riboflavin hydrochloride, 5.0; nicotinic acid, 5.0; dl-calcium pantothenate, 5.0; vitamin B₁₂, 0.1; p-aminobenzoic acid, 5.0; and lipoic acid, 5.0. The medium was gassed with either N₂-CO₂ (80:20, vol/vol) or H₂-CO₂ (80:20, vol/vol). The pH was adjusted to 7.0 to 7.1 with solid KHCO₃ (6 g/liter). The sterilized medium was reduced just before inoculation with Na₂S · 9H₂O (0.15 g/liter) and cysteine hydrochloride (0.3 g/liter). Energy sources were fructose (20 mM), methanol (12 mM), or H₂-CO₂. Growth experiments were done in either 500-ml (H₂-CO₂ as substrate) or 250-ml (fructose or methanol as substrate) bottles with 50 ml of medium. Growth was followed by measuring the A₆₀₀.

Preparation of cell suspensions. For resting-cell experiments with methanol, the organisms were grown on 12 mM methanol. In all other cases, they were grown on 20 mM fructose. Cultures were harvested at the end of the exponential growth phase by centrifugation (23,500 × g, 20 min, 4°C) under anaerobic conditions and washed twice with 20 mM imidazole–HCl buffer, pH 6.7, containing 50 mM KCl, 5 mM MgSO₄, and 6 mM dithiothreitol (DTT). The cells were resuspended in the same buffer to a final protein concentration of 15 to 20 mg/ml (growth on methanol) or 25 to 30 mg/ml (growth on fructose) under an atmosphere of nitrogen. This suspension was used immediately for the experiments. The protein concentration of the cell suspension was determined by the method of Schmidt et al. (23). All manipulations were done under strictly anaerobic conditions in an anaerobic chamber (M. D. H. GmbH, Rüsselsheim, Federal Republic of Germany).

Experiments with cell suspensions. The experiments with
cell suspensions were performed in 115-ml bottles; they contained, in a final volume of 10 ml, 50 mM imidazole buffer, 20 mM KCl, 5 mM MgSO₄, 6 mM DTT, and cells as given in the figure legends. The buffer was adjusted to pH 7 by titration with HCl for all experiments without CO₂ in the gas phase and by gassing with N₂-CO₂ (80:20, vol/vol) for all those experiments which contained CO₂ in the gas phase. NaCl, fructose, methanol, or formaldehyde was added as indicated for each experiment. Incubations were done at 30°C on a rotary shaker (70 rpm) under an atmosphere of N₂-CO₂ (80:20, vol/vol; 111 kPa), H₂-CO₂ (80:20, vol/vol; 111 kPa), or H₂-CO₂ (92:8, vol/vol; 121 kPa). To determine H₂ formation from CO, the experiments were performed in 58-ml bottles under an atmosphere of N₂ (111 kPa). Additions were made as indicated. Monensin was added as an ethanolic solution; the control received only the solvent. The formaldehyde solution was prepared by the method of Blaut et al. (3). The experiments were started by the injection of 0.3 to 0.5 ml of the concentrated cell suspension. At the times indicated, samples (0.4 ml) were withdrawn by syringe and centrifuged, and the supernatant was analyzed for acetate and fructose by enzymatic assays (2, 6). Rates of acetate formation were calculated from sampling periods of at least 45 min. H₂ was measured by gas chromatography (17). The external sodium concentration was determined with a sodium electrode (Orion Research AG, Küssnacht, Switzerland) connected to an ion meter (Orion Research AG).

**Measurements of Na⁺ movements.** To determine the substrate-dependent movement of Na⁺ across the membrane, cell suspensions in the above imidazole buffer plus 20 mM NaCl (final volume, 1.6 ml) were incubated in 8-ml bottles under an atmosphere of H₂-CO₂ (80:20, vol/vol; 127 kPa) or N₂-CO₂ (80:20, vol/vol; 127 kPa) on a rotary shaker for 10 min at 30°C. At time zero, 2 μl of carrier-free ²²NaCl (0.7 μCi/μl) was added. Samples (100 μl) were withdrawn by syringe as indicated in the legend to Fig. 4, transferred to membrane filters (25 mm in diameter; pore size, 0.45 μm; Sartorius, Göttingen, Federal Republic of Germany) and washed four times with 1 ml of buffer. The filters were counted in a liquid scintillation counter with 10 ml of Rialuma (J. T. Baker B. V., Deventer, Holland). Unspecific binding of ²²Na⁺ on the filter was reduced by overnight preincubation of the filters in 20 mM NaCl. The intracellular Na⁺ concentration was calculated after correction for unspecific binding of ²²Na⁺ on the filters and with an intracellular volume of 3.2 μl/mg of protein (R. Boenigk, Diploma thesis, Universität Göttingen, 1988).

**Chemicals and gases.** Imidazole was purchased from Serva, Heidelberg, Federal Republic of Germany. Enzymes were from Boehringer, Mannheim, Federal Republic of Germany. Monensin was purchased from Sigma.
Taufkirchen, Federal Republic of Germany. Propylidode was from Merck, Darmstadt, Federal Republic of Germany. 22NaCl was from New England Nuclear Corp., Dreieich, Federal Republic of Germany. Gases were from Messer-Griesheim, Kassel, Federal Republic of Germany.

RESULTS

Sodium dependence of growth on fructose, H2-CO2, or methanol. Cells of A. woodii were transferred to medium supplemented with various amounts of NaCl. Growth on fructose under an atmosphere of N2-CO2 was stimulated by the presence of NaCl in the medium; the organisms reached a maximal A600 of 2.5 at 20 mM NaCl and grew with a doubling time of 4.2 h, whereas an A600 of 1.8 and a doubling time of 7.1 h were reached at 0.2 mM NaCl (Fig. 2). The ratio of acetate formed to fructose consumed decreased from 2.7 to 2.1 when the NaCl concentration was lowered from 20 to 0.2 mM. H2 production was stimulated in the absence of NaCl; 5 and 12 μmol of H2 were produced from 1 mmol of fructose at 20 and 0.2 mM NaCl, respectively. Small amounts of ethanol were produced (approx. 25 μmol of ethanol at 20 mM and 100 μmol at 0.2 mM NaCl per mmol of fructose). Formate and succinate were not found in detectable amounts. Contrary to growth on fructose, growth on H2-CO2 was strictly dependent on the presence of NaCl. At 0.7 mM NaCl, no growth occurred; higher NaCl concentrations resulted in increased growth rates, and the maximum rate was observed at 20 to 30 mM NaCl (Fig. 3). The concentration of Na+ required for one-half of the maximum growth rate was 6 mM. The minimum doubling time at NaCl concentrations above 20 mM was 5.5 h. A dependence on NaCl similar to that for growth on H2-CO2 was observed with methanol as the substrate (data not shown).

Localization of the sodium-dependent step in acetogenesis. To characterize the sodium dependence of growth and acetate formation more precisely, experiments with resting cells were performed. Cell suspensions of A. woodii were

FIG. 2. (A) Growth (△ and △) of A. woodii and (B) effect of NaCl concentration on fructose (○, ○) and acetate (□, □) formation. A 50-ml amount of medium with 20 mM fructose was inoculated with 2 ml of a log-phase culture of A. woodii and incubated without shaking at 30°C. Solid symbols, 20 mM NaCl; open symbols, 0.2 mM NaCl.
TABLE 1. Effect of Na$^+$ on acetate formation from various substrate combinations

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Sodium*</th>
<th>Acetate formation (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose, N$_2$-CO$_2$</td>
<td>+</td>
<td>72.7</td>
</tr>
<tr>
<td>H$_2$-CO$_2$</td>
<td>-</td>
<td>51.2</td>
</tr>
<tr>
<td>Methanol, N$_2$-CO$_2$</td>
<td>+</td>
<td>21.6</td>
</tr>
<tr>
<td>Methanol, H$_2$-CO</td>
<td>-</td>
<td>37.8</td>
</tr>
<tr>
<td>Formaldehyde, N$_2$-CO$_2$</td>
<td>+</td>
<td>60.9</td>
</tr>
<tr>
<td>Formaldehyde, H$_2$-CO</td>
<td>-</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Concentrations of substrates were: fructose, 10 mM; methanol, 5 mM; formaldehyde, 2.5 mM; H$_2$-CO$_2$ (80:20, vol/vol; 111 kPa); N$_2$-CO$_2$ (80:20, vol/vol; 111 kPa); H$_2$-CO (92:8, vol/vol; 121 kPa). The protein content of the final cell suspensions was 0.7 to 1.5 mg/ml.

* +, 20 mM NaCl added; −, no sodium salts added and the sodium concentrations determined were lower than 0.2 mM.

incubated in buffer under an atmosphere of H$_2$-CO$_2$. Acetate formation started immediately after injection of cells and proceeded with a linear rate for at least 3 h. As was observed with growing cultures, acetate formation was dependent on the presence of NaCl. At 20 mM NaCl, an 8- to 10-fold stimulation of acetate formation was observed compared with the rate without the addition of sodium salts (Table 1). Na$_2$SO$_4$ but neither KCl nor choline chloride could substitute for NaCl, indicating that sodium ions are essential for growth as well as for acetate formation. Lithium ions could substitute for sodium ions. The concentration of Na$^+$ required for one-half of the maximum rate of acetogenesis was 4 mM; the corresponding concentration of Li$^+$ was approximately 10-fold higher. In contrast to the observed strong Na$^+$ dependence for H$_2$-CO$_2$, acetate formation from fructose was only slightly dependent on sodium ions. The rate of acetate formation in the absence of Na$^+$ was approximately two-thirds (70.4%) of the maximal rate at 20 mM NaCl.

In view of this difference, Na$^+$ must be primarily required for autotrophic synthesis of acetate. In order to localize the Na$^+$-dependent step during autotrophic acetate formation, experiments with various substrate combinations were conducted (Table 1).

A. woodii is able to convert formaldehyde or methanol to acetate in the presence of molecular hydrogen and carbon monoxide or in the presence of carbon dioxide (5). The sodium dependence of these fermentations was studied. Resting cells of A. woodii incubated with formaldehyde in the presence of 20 mM NaCl under an atmosphere of H$_2$-CO produced acetate with a linear rate for 2 h. Without Na$^+$, very little acetate was formed. Acetate formation from methanol under H$_2$-CO, however, was sodium independent. When the gas atmosphere was changed to N$_2$-CO$_2$, a strict Na$^+$ requirement was seen with both substrates. An effect of pH caused by CO$_2$ could be excluded, because the initial pH in the experiments was always 7. With formaldehyde under H$_2$-CO, A. woodii produced more acetate than would be expected from the stoichiometric conversion of formaldehyde plus CO, indicating that carbon monoxide must be converted to additional acetate by disproportionation to H$_2$ plus CO$_2$. This coconsumption was not observed with methanol under H$_2$-CO. From H$_2$-CO (92:8, vol/vol) alone, no acetate was formed.

It has been shown that the reduction of CO$_2$ to CO as catalyzed by carbon monoxide dehydrogenase is an energy-dependent reaction (5). Therefore, it was of interest to study the effect of Na$^+$ on this reaction. Resting cells of A. woodii were incubated under an atmosphere of N$_2$ in the presence of 10 mM propylidode, which is known to inhibit acetate formation from CO (9). After addition of CO (final concentration, 98.8% N$_2$, 1.2% CO), the cells formed H$_2$ from CO irrespective of the presence or absence of NaCl at rates between 50 and 60 nmol/min per mg of protein. Acetate formation did not occur. These experiments are not in favor of a possible role of Na$^+$ as the driving force for CO$_2$ reduction. This interpretation is supported by the observation that the sodium dependence of acetate formation from methanol under N$_2$-CO$_2$ could be overcome by the addition of H$_2$ (data not shown). Therefore, the sodium dependence must be connected to methanol oxidation which has to occur if no external H donor is available for acetate synthesis from methanol plus CO$_2$. Acetate formation in the absence of NaCl from methanol plus CO$_2$ plus H$_2$ shows unequivocally that the reduction of CO$_2$ to CO with H$_2$ does not require Na$^+$.

FIG. 4. Sodium extrusion as a result of acetogenesis from H$_2$-CO$_2$. One cell suspension (C) was incubated under an atmosphere of N$_2$-CO$_2$. At the time indicated by the arrow, 5 ml of H$_2$ was added. The other cell suspension (B) was incubated under H$_2$-CO$_2$. For experimental details, see Materials and Methods. Protein content of the cell suspension: 3.2 mg/ml Na$^+$, Intracellular sodium concentration.
mM NaCl was inhibited. At 0.2 mM NaCl, however, acetogenesis was transiently stimulated (Fig. 5B).

DISCUSSION

The catabolic flexibility of *Acetobacterium woodii* allows the reaction site at which sodium ions are required to be narrowed down. When these organisms ferment fructose, Na⁺ is only stimulatory. However, growth in low sodium is accompanied by a shift of the acetate-fructose ratio from 2.7 to 2.1. This shift is indicative of a possible role of Na⁺ not in glycolysis but in reduction of CO₂ to acetate, a conclusion which is supported by the observed strict dependence on Na⁺ of acetate formation from CO₂ plus H₂. A further narrowing of the Na⁺-requiring reaction was made possible by the use of formaldehyde and methanol as substrates. Acetate formation from methanol plus H₂-CO₂ was Na⁺ dependent. The fermentation of methanol involves the methylation of a corrinoid protein by a transferase reaction (29), subsequent carboxylation, and formation of acetate via acetyl-coenzyme A and acetyl phosphate (Fig. 1). Apparently none of these reactions require Na⁺. However, when methanol is replaced by formaldehyde, an Na⁺ requirement was detectable. This indicates that Na⁺ plays a role in the conversion of methylene-tetrahydrofolate, which is formed from formaldehyde and tetrahydrofolate (14), to methyltetrahydrofolate. The corresponding reductase has recently been shown to be membrane bound (11), which is in accordance with a possible function of this enzyme as a primary sodium pump. Acetogenesis from methanol under N₂-CO₂ was strictly Na⁺ dependent. Under these conditions, methyl groups are expected to be oxidized through the same enzymes which are involved in the reductive pathway (16). However, it should be mentioned in this connection that evidence has been obtained for a novel methanol dehydrogenase with *Clostridium thermoautothrophicum* and *Eubacterium limosum* (7), which contains the coenzyme pyrroloquinoline quinone.

*A. woodii* is able to create a rather steep Na⁺ gradient across the cytoplasmic membrane. Intracellular Na⁺ has been found to be 20 to 40 times lower than extracellular Na⁺. During incubation under N₂-CO₂, this gradient is slowly dissipated, but it is immediately reestablished when the gas atmosphere is replaced by H₂-CO₂. Monensin, which functions as a very effective Na⁺/H⁺ antiporter, dissipates this gradient and simultaneously inhibits acetogenesis. If the NaCl concentration is high, this inhibition is instantaneous. At low sodium, stimulation of the low rate of acetogenesis is observed first, followed by inhibition. Such a stimulation by monensin at low NaCl concentrations was also seen in methanogenesis from H₂-CO₂ by *Methanobacterium thermoautotrophicum* (25) and *Methanosarcina barkeri* (V. Müller, unpublished results). A possible explanation is that in low Na⁺, the processes of methanogenesis and acetogenesis are limited by the availability of intracellular Na⁺ for the sodium pump. Na⁺ is then brought into the cells by the antiporter activity of monensin. After some time, however, the cytoplasmic membrane is deenergized, probably resulting in a drop of the energy charge so that ATP is not available any more for the ATP-requiring steps of acetogenesis. This conclusion is supported by the fact that inhibition by monensin of acetate formation from formaldehyde under H₂-CO₂ in high sodium is much weaker and the transient stimulation at low sodium is stronger than that observed from H₂-CO₂ (data not shown).

The importance of sodium bioenergetics in methanogenic bacteria was discovered when methanogenesis from methanol by *Methanosarcina barkeri* was studied (18, 21). It was then shown that oxidation of methyl groups to methane groups is driven by Na⁺ influx and that the sodium gradient required for this reaction is generated by an active Na⁺/H⁺ antiporter (19). When methanogenesis from H₂ plus CO₂ and from H₂ plus formaldehyde was studied, it could be unequivocally shown that the reverse reaction—the reduction of methane groups to methyl groups—is coupled to sodium extrusion, and it is this reaction which apparently has its parallel in acetogenesis as carried out by *A. woodii*.

Further characterization of the mechanism for sodium extrusion and utilization of the sodium gradients is now in progress in our laboratory.

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LITERATURE CITED