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Electron Transport and Electrochemical Proton Gradient in Membrane Vesicles of *Clostridium thermoautotrophicum*

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Membrane vesicles of *Clostridium thermoautotrophicum* containing carbon monoxide dehydrogenase generated a proton motive force when exposed to CO. This proton motive force, with a value of −140 mV, consisted of only an electrical potential at pH 7.5 and above and of an electrical potential and pH gradient at a lower pH. The proton motive force drove the uptake of L-alanine by the vesicles to a concentration of 300 times that of the medium.

Clostridium thermoautotrophicum is an acetogenic bacterium. It forms acetate as the main product, growing either on sugars or on C_1-compounds such as CO_2-H_2, CO, methanol-CO_2, or formate. The specialized pathway used by acetogens has been summarized in several reviews (6, 11, 22), but their energy metabolism has not received much attention. Unusually high growth yields when growing heterotrophically (1, 20) and the ability to grow autotrophically on C_1-compounds (6, 11, 21) indicate that they generate energy by a chemiosmotic process (14) coupled to the synthesis of acetate (6, 11, 19). This process requires membrane-associated electron transport with simultaneous pumping of protons and an ATP-synthesizing H^+-ATPase. H^+-ATPase is present in *Clostridium thermoaceticum* and *C. thermoautotrophicum* (8, 13; D. M. Ivey and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K-33, p. 208), and electron transport in membrane vesicles of *C. thermoautotrophicum* involves a flow of electrons from carbon monoxide, H_2, or NADH via a flavoprotein to two *b*-type cytochromes (7; J. Hugenholtz and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K-153, p. 232; D. M. Ivey, Ph.D. dissertation, University of Georgia, Athens, 1987). Here we report on the generation of proton motive force (Δp) by CO-driven electron transport in membrane vesicles of *C. thermoautotrophicum*, and we demonstrate that this Δp serves as an energy source for the uptake of L-alanine.

C. thermoautotrophicum 701/5 was grown in an anaerobic medium on glucose under CO_2 atmosphere at 59°C and pH 6.5 (12). Membrane vesicles were prepared as described previously (7, 15). Protein (4) and carbon monoxide dehydrogenase activity (16) were assayed as previously published. Hydrogenase activity was determined with 10 mM methyl viologen as electron acceptor in 0.1 mM glycine, pH 9.0, under anaerobic conditions. One unit of enzyme activity is equal to the oxidation of 1 μmol of CO or H_2 per min. Labeled chemicals were obtained from Amersham Corp. (Arlington Heights, Ill.) and from Dupont, NEN Research Products (Boston, Mass.).

The electrical potential (ΔΨ) was calculated from the distribution of tetraphenyl phosphonium ([^{14}C]TPP$^+$) across the membranes (18), which was measured under anaerobic conditions by the filtration technique described by Kaback (9). [^{14}C]TPP$^+$ (31.4 Ci/mol) was added to a final concentration of 30 μM. At time zero the artificial electron acceptor was added; methyl viologen (10 mM), benzyl viologen (10 mM), methylene blue (5 mM), or ferricyanide (10 mM) was used. As a control for binding of TPP$^+$ to the membranes, TPP$^+$ uptake was measured in the absence of electron acceptor or electron donor. The uncoupler CCCP (*m*-chlorocarbonyl-cyanide phenylhydrazone) (10 μM) or the ionophores valinomycin (a K^+ channel) (10 μM) and nigericin (2 μM) were added 5 min prior to the addition of electron acceptor. The proton gradient (ΔpH) was assayed similarly by determining the distribution of the weak acid [^{14}C]salicylate (50.4 Ci/mol) across the membrane (2, 18), using a concentration of 16 μM. Accumulation ratios of TPP$^+$ and salicylate were calculated by using an internal volume of 3.0 μl per mg of membrane protein, which was determined by the method of Konings and Freese (10). The uptake of L-[^{14}C]alanine (171 Ci/mol, 5.6 μM) into the membrane vesicles was measured as described for the uptake of TPP$^+$ and salicylate. Carbon monoxide was used as electron donor and ferricyanide (10 mM) was used as electron acceptor in 50 mM potassium phosphate, pH 7.0.

A Δp was generated in membrane vesicles by carbon monoxide oxidation in the presence of ferricyanide. With methyl viologen, benzyl viologen, or methylene blue as electron acceptor, carbon monoxide was oxidized but a Δp was not generated. The dyes accept electrons directly from the oxidation of carbon monoxide (16) without actual electron transport and resulting proton pumping. In the absence of ferricyanide, no uptake of TPP$^+$ or salicylate was observed. The Δp was generated within 1 min at 50°C and after

TABLE 1. Membrane potential (Δψ) developed in membrane vesicles prepared from *C. thermoautotrophicum* with CO as electron donor and ferricyanide as electron acceptor[a]

pH	Expt 1 (no addition)		Expt 2 (plus nigericin)		Z ΔpH (mV)[b]
	cpm[c]	mV	cpm	mV	
7.5	29,890	−126	30,010	−126	0
7.0	26,200	−122	31,340	−127	5
6.5	24,680	−121	32,080	−128	7
6.0	14,830	−106	42,310	−137	31

[a] Incubation mixtures contained membrane vesicles corresponding to 45 μg of protein with an internal volume of 3 μl/mg of protein; 50 mM potassium phosphate buffer; 10 mM ferricyanide; 30 μM [^{14}C]TPP$^+$, containing a total of 197,200 cpm; and 2 μM nigericin when indicated, in a total volume of 100 μl.

[b] Z ΔpH is the difference of the Δψ values in experiments 1 and 2. Δψ was calculated by the formula $\Delta\Psi = -59 \times [\log (TPP^+_{inside}/TPP^+_{outside})]$.

[c] Accumulated inside the membrane vesicles after deduction of nonspecific binding of [^{14}C]TPP$^+$.

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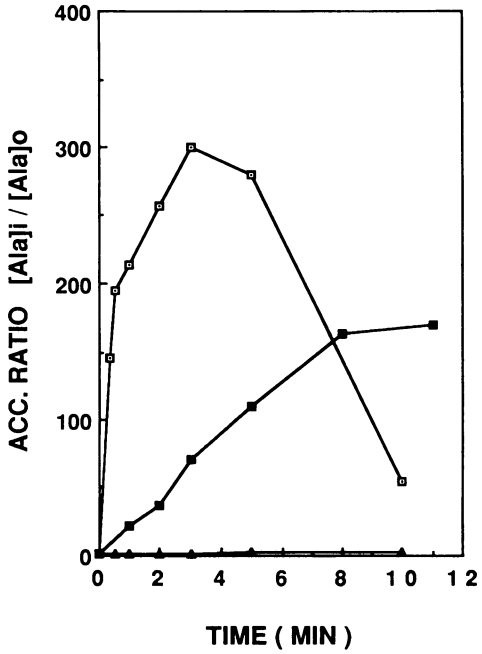

FIG. 1. Time-dependent uptake of L-alanine by membrane vesicles of *C. thermoautotrophicum* at 25°C (■) and at 50°C (□). L-Alanine uptake in the presence of 10 μM CCCP or in the absence of carbon monoxide is also shown (▲). The concentration of L-alanine was 5.6 μM, and the amount of membrane protein used in each assay was 40 μg in 50 mM potassium phosphate, pH 7.0. Time zero is the time of addition of ferricyanide. Acc., Accumulation; [Ala]i, alanine inside; [Ala]o, alanine outside.

3 min at 25°C. It was maintained for up to 3 and 10 min at 50 and 25°C, respectively, but collapsed thereafter due to the depletion of oxidized ferricyanide.

The value of Δp was dependent on the activity of carbon monoxide dehydrogenase in the membrane vesicles, which could vary from 2 to 20 U per mg of protein (measured at pH 7.6 and 50°C). A minimum carbon monoxide dehydrogenase activity of ≈4 U/mg was necessary for the generation of a Δp. Table 1 shows results for a batch of membranes with carbon monoxide dehydrogenase activity of 15 U/mg. At 25°C a $\Delta\Psi$ of −126 mV was generated at pH 7.5. The $\Delta\Psi$ changed with decreasing pH to −106 mV at pH 6.0, and at lower pH values it reached insignificant values. The $\Delta\Psi$ values at pH 7.0 and 7.5 were similar when measured in potassium phosphate or Tris buffer.

A pH gradient seemed to be absent at all external pH values. However in the presence of nigericin, which acts as an artificial K^+/H^+ antiporter, a significant increase in the accumulation of TPP^+ was observed (Table 1), indicating that the original pH gradient had been converted into a $\Delta\Psi$. As would be expected, no pH gradient was observed at pH 7.5, and the largest pH gradient was found at pH 6.0 (31 mV = 0.53 pH units). No pH gradient could be measured at pH values below 6.0. The addition of CCCP had the same effect as leaving out carbon monoxide or ferricyanide; no Δp was generated.

Δp generation with H_2 as electron donor was also studied in the membrane vesicles with either methyl viologen or benzyl viologen as electron acceptor. Methylene blue and ferricyanide could not be used, since they inhibit hydrogenase activity. No Δp formation could be measured below pH 7.5 with either of the electron acceptors. At pH 8.0 and 50°C, a small but significant Δp (−20 to −30 mV) was found with benzyl viologen as electron acceptor. The hydrogenase reaction with methyl viologen was too slow to generate a Δp. These results were observed only in membrane vesicles with at least 4 U of hydrogenase activity per mg (measured at pH 9.0 and 50°C). This Δp generation is not a result of electron transport, since benzyl viologen accepts electrons directly from hydrogenase. This indicates that the hydrogenase is located on the outside of the cytoplasmic membrane. The low values of the Δp are due to proton leakage through the membrane. A minimum proton-pumping activity is required to overcome this leakage and generate a Δp. This explanation is supported by the observations that membranes with lower hydrogenase activity (at pH <8.0) and low CO dehydrogenase activity (<4 U/mg) are unable to generate a Δp.

In the presence of CO and ferricyanide, L-alanine, glycine, and L-serine but not L-glutamate, L-leucine, pyruvate, lactate, or acetate were actively transported into the membrane vesicles. L-Alanine was accumulated up to 300-fold in the vesicles. These maximum uptake levels were reached after approximately 10 min at 25°C and after less than 2 min at 50°C (Fig. 1). The calculated chemical gradient for alanine (−159 mV) was slightly higher than the measured driving force (Δp = −127 mV) (Table 1). This was probably due to underestimation of both the $\Delta\Psi$ and the ΔpH values caused by rapid efflux of TPP^+ from the membrane vesicles during the $\Delta\Psi$ measurements (17) and an incomplete conversion of the ΔpH into the $\Delta\Psi$ upon addition of nigericin. The uptake of L-alanine was inhibited 10% by nigericin, 80% by valinomycin, and totally by CCCP. These results indicate that both ΔpH and $\Delta\Psi$ are driving forces for L-alanine uptake. In the absence of electron donor or electron acceptor, L-alanine did not accumulate inside the vesicles; it merely equilibrated over the membrane.

Results presented here give evidence that energy is generated by membrane-associated electron transport in the obligate anaerobe *C. thermoautotrophicum*. This energy, in the form of a Δp, can drive the synthesis of ATP (8) and, as

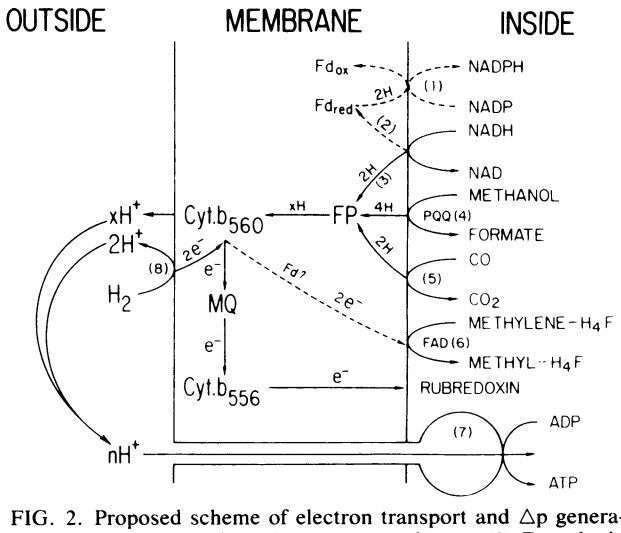

FIG. 2. Proposed scheme of electron transport and Δp generation in the membrane of *C. thermoautotrophicum*. (1) Ferredoxin (Fd) NADP:oxidoreductase; (2) ferredoxin NAD:oxidoreductase; (3) NADH dehydrogenase; (4) methanol dehydrogenase; (5) carbon monoxide dehydrogenase; (6) methylenetetrahydrofolate reductase; (7) F_1F_0-H^+-ATPase; (8) hydrogenase. FAD, Flavin adenine dinucleotide; FP, flavoprotein; MQ, menaquinone; PQQ, pyrroloquinoline quinone.

shown here, the uptake of amino acids, as exemplified with L-alanine. The measured values of the Δp in the membrane vesicles (Table 1) are similar to those reported for whole cells of glucose-grown *C. thermoaceticum* (2). They are high enough to explain growth of acetogens on carbon monoxide and may also explain the unusually high growth yields for acetogens on sugars. Δp generation by oxidation of CO has been reported previously for whole cells of the acetogen *Acetobacterium woodii* (3).

The electron flow and proton pumping, as we understand it, are shown schematically in Fig. 2. Electron donors can be CO, H_2, NADH, or methanol (D. Winters-Ivey, Ph.D. thesis, University of Georgia, Athens, 1987). In all cases, the cytochromes b_{560} (E_0' − 200 mV) and b_{556} (E_0' − 48 mV) are reduced, either directly (H_2) or via an unknown flavoprotein (CO and NADH). The physiological electron acceptor is, presumably, methylenetetrahydrofolate (7). It is reduced to methyltetrahydrofolate, which acts as a donor for the methyl group in acetate synthesis. Methylenetetrahydrofolate reductase is on the inside of the cytoplasmic membrane (7), and inside-out membrane vesicles will be required to study the electron flow to methylenetetrahydrofolate. Several aspects of electron transport in *C. thermoautotrophicum* remain obscure, among them the role of the high-potential side chain of the electron flow via menaquinone to cytochrome b_{556} (and possibly to rubredoxin), the mechanism of NADPH generation with a possible involvement of a membrane-associated ferredoxin (5), and the transfer of electrons from the pyrroloquinoline quinone-dependent methanol dehydrogenase.

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