

Carbon Monoxide-Driven Electron Transport in *Clostridium thermoautotrophicum* Membranes

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Membrane vesicles of *Clostridium thermoautotrophicum* prepared by osmotic lysis after lysozyme treatment contained carbon monoxide dehydrogenase and methylenetetrahydrofolate dehydrogenase with specific activities three- to fourfold higher than the specific activity of the cytoplasm. The membrane-associated carbon monoxide dehydrogenase mediated the reduction with CO or the oxidation with CO₂ of *b*-type cytochromes and other electron carriers in the membrane.

Acetogenic bacteria, such as *Clostridium thermoautotrophicum*, grow heterotrophically on several sugars and autotrophically on H₂-CO₂, CO, and methanol-CO₂ (3, 23; D. W. Ivey, L. G. Ljungdahl, and J. Wiegel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K66, p. 182). When growing on these substrates, acetogens synthesize acetate from two C₁ compounds via an intricate pathway involving several tetrahydrofolate (H₄folate) enzymes, a corrinoid protein, a nickel-containing carbon monoxide dehydrogenase, and a tungsten-selenium formate dehydrogenase (7, 12, 24).

The energy metabolism of acetogens is essentially unknown. When fermenting sugars, acetogens derive their metabolic energy mostly by substrate-level phosphorylation. However, growth yields of *Clostridium thermoaceticum* (1) and *Acetobacterium woodii* (21) on glucose are higher than can be accounted for by substrate-level production of ATP. During autotrophic growth on H₂-CO₂, no net substrate-level phosphorylation occurs. Acetyl phosphate is formed, and it may yield ATP, but this ATP is needed for the synthesis of formyl-H₄folate from formate and H₄folate. Thus, an alternative way of energy generation must exist. Several authors have suggested that this may involve cytoplasmic-membrane-associated chemiosmosis and have referred to the presence of membrane-associated electron transfer proteins such as cytochromes and menaquinones in *C. thermoaceticum* and *Clostridium formicoaceticum* (8).

Recent findings indicate that chemiosmotic energy transduction occurs in acetogens. *C. thermoaceticum* and *C. thermoautotrophicum* contain H⁺-ATPases that catalyze a pH-gradient-driven ATP synthesis (9, 15; D. M. Ivey and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K33, p. 208). Generation of a proton motive force in whole cells of *A. woodii* has also been observed in association with the oxidation of CO (4). It has been predicted that the enzymes carbon monoxide dehydrogenase and methylene-H₄folate reductase are involved in electron transport reactions (7, 20). But all enzymes of the acetate synthesis pathway have been found to be soluble (12, 24), an indication that they are not associated with the cytoplasmic membrane. However, in this report we present evidence that carbon monoxide dehydrogenase and methylene-H₄folate reductase are associated with the cytoplasmic membrane of *C. thermoautotrophicum* and that carbon monoxide dehydrogenase mediates the CO-dependent reduction of components of the membrane, including two *b*-type cytochromes.

C. thermoautotrophicum 701/5 was grown in anaerobic medium on glucose (14) under CO₂ atmosphere at 59°C at pH 6.5 and harvested during exponential growth (*A*₆₆₀, 1.5). Membrane vesicles were prepared by lysozyme treatment by the method of Kaback (11) modified by Otto et al. (16). A lysozyme concentration of 0.8 mg/ml and a temperature of 47°C were used during lysis of the cells. The membranes were washed with 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM dithiothreitol and all metals present in the growth medium (14). The membranes were then stored at 4°C in 50 mM potassium phosphate buffer containing 3 mM dithiothreitol and 20% glycerol. Membrane vesicles were made permeable with the addition of 1% toluene. Cell extracts and membranes were also prepared with the French pressure cell as described previously (9). Anaerobic conditions were applied throughout all procedures by using a type B anaerobic chamber (Coy Laboratory Products).

H⁺-ATPase (9), carbon monoxide dehydrogenase (17), hydrogenase (18), formate dehydrogenase (13), methylene-H₄folate reductase (2), and formyl-H₄folate synthetase (19) were assayed as described previously. Protein was measured by the rose bengal dye-binding assay (5) in the presence of 0.2% Triton X-100 to solubilize the membrane proteins. Bovine serum albumin was used as the standard. Enzyme units are in micromoles of substrate converted per minute, and specific activity is in units per milligram of protein. Metals were determined in membranes after they had been repeatedly washed with potassium phosphate buffer by plasma emission spectroscopy (10). Difference spectra (with and without CO and dithionite) were recorded by using anaerobic cuvettes (13) with an Aminco DW-2 UV-Vis spectrophotometer (American Instrument Co., Silver Spring, Md.). CO was added to the cuvettes either by direct gassing or by the addition of CO-saturated phosphate buffer.

Intact membranes obtained by lysozyme treatment displayed low H⁺-ATPase activity. After the vesicles were made permeable with toluene, this activity increased four- to fivefold (Table 1). This increase indicated that the membranes had retained their *in vivo* (right side out) orientation at 77%. When the membranes were frozen and subsequently thawed or when the cells were lysed at 30°C, 50% or more of the membranes showed a reverse (inside out) orientation (data not shown). Membranes stored at 4°C in the presence of 20% glycerol under anaerobic conditions maintained the membrane-associated enzyme activities for 1 month.

Major parts of the total cellular activities of carbon monoxide dehydrogenase (>80%) and methylene-H₄folate

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TABLE 1. Enzyme activities in cell extracts, the cytoplasmic fraction, and membrane preparations of *C. thermoautotrophicum*^a

Enzyme	Cell extract ^b	Cytoplasmic fraction ^c	Membranes ^c	
			Intact	Permeable
H ⁺ -ATPase	ND ^d	ND	0.37	1.63
Carbon monoxide dehydrogenase	6.0	4.6	18.0	14.6
Methylene-H ₄ folate reductase	0.45	0.38	0.48	1.33
Formyl-H ₄ folate synthetase	11.1	10.2	0.04	0.06

^a Figures are in enzyme units per milligram of protein.

^b Prepared with French pressure cell.

^c Prepared by lysozyme treatment.

^d Not determined.

reductase (about 70%) were retained in the membrane fraction after osmotic lysis of the cells. Up to 50 and 25% of these activities, respectively, became soluble with the first washing of the membranes, but repeated washing did not remove any more of the membrane-associated enzyme activities (data not shown). The specific activities of the two enzymes in the washed membranes were threefold higher than in cell extracts prepared with a French pressure cell and fourfold higher than in the cytoplasmic fraction released on osmotic lysis of the cells (Table 1). In contrast, the specific activity of formyl-H₄folate synthetase, which is considered a typical cytoplasmic enzyme, was about 150-fold lower in the membrane fraction than in the cytoplasmic fraction (Table 1). These data demonstrate that carbon monoxide dehydrogenase and methylene-H₄folate reductase are associated with the cytoplasmic membrane in *C. thermoautotrophicum*. Treatment of the membrane vesicles with toluene did not affect the activity of carbon monoxide dehydrogenase. This was expected since CO is a membrane-permeable substrate. However, the activity of methylene-H₄folate reductase increased almost threefold after toluene treatment of the membranes, indicating that this enzyme was located on the cytoplasmic side of the membrane. Hydrogenase and formate dehydrogenase were not found in the membrane preparations obtained with lysozyme. Membranes prepared with the French pressure cell also lacked these enzymes, as well as carbon monoxide dehydrogenase and methylene-H₄folate reductase. Our results with carbon monoxide dehydrogenase are similar to those obtained by Wakim and Uffen (22). Using the French pressure cell, they found with *Rhodospseudomonas gelatinosa* that carbon monoxide dehydrogenase was solubilized to more than 90% but was associated with the membrane on osmotic lysis of spheroplasts.

The amount of nickel in French-press membrane preparations (0.17 nmol/mg of protein) was much lower than in membranes obtained by the lysozyme treatment (0.99 nmol/mg of protein). This finding correlates with the presence of carbon monoxide dehydrogenase in the latter.

Incubation of the membranes with CO had a distinct effect on their visible spectra. The difference spectra (+CO/+O₂) showed patterns typical of the two *b*-type cytochromes that have been identified in *C. thermoaceticum* and in *C. thermoautotrophicum* (8; D. M. Ivey and L. G. Ljungdahl, unpublished results) with α peaks at 556 and 560 nm, β peaks at 525 and 530 nm, and Soret peaks at 420 and 430 nm (Fig. 1). The spectral changes were the results of reduction mediated by carbon monoxide dehydrogenase and not by a direct reaction of the cytochromes with CO, since the addition of dithionite resulted in the same reduced cyto-

chrome spectrum. In addition to the cytochromes, other membrane components seemed to be reduced, as indicated by a loss of absorbance between 435 and 500 nm. The reduction of the different components occurred in a sequence. This was observed when the reduction was carried out at 20°C (Fig. 1). The cytochromes were reduced before the material with absorption in the 435- to 500-nm region. At 50°C, no sequence in reduction was observed; within 30 s, all components were reduced. A longer incubation with CO resulted in a spectral shift of the Soret peak to below 420 nm and in a decrease of the α peak, indicating a direct reaction between the reduced cytochromes and CO (data not shown).

Spectroscopic examination of the membranes at -196°C allowed the distinction of the two cytochromes (Fig. 2). One of the cytochromes with absorption peaks at 554, 525, and 424 nm appeared to be reduced almost completely in the membranes as prepared. This *b*-type cytochrome has a relatively high potential (-48 mV) (Ivey and Ljungdahl, unpublished results). On reduction with CO, the spectrum was dominated by the second *b*-type cytochrome present in *C. thermoautotrophicum*. This cytochrome has a low redox potential (-200 mV) and has absorption peaks at 559, 530, and 428 nm. It, but not the high-potential cytochrome, was at least partly oxidized when CO-reduced membranes were treated with CO₂ (Fig. 2).

As shown by Diekert et al. (4), oxidation of CO in *A. woodii* is coupled to energy generation. Our finding that carbon monoxide dehydrogenase in *C. thermoautotrophicum* is membrane bound agrees with this finding. It has been suggested also that the reduction of methylene-H₄folate

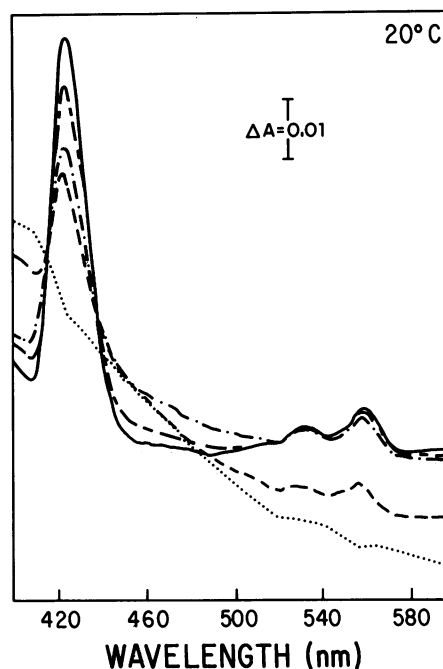


FIG. 1. Difference spectra (reduced versus oxidized) of membrane vesicles of *C. thermoautotrophicum* recorded anaerobically at 20°C with CO as a reductant. The membranes were not exposed to CO (-----) or were exposed for 30 (- . - . -), 120 (-----), and 300 s (—) before the spectra were recorded. The reference cuvette contained membranes oxidized with air. The dotted line (.) is the uncorrected base line taken before the reference sample was oxidized with air. The reactions were done with 0.4 mg of protein per ml in 50 mM potassium phosphate (pH 7) containing 3 mM dithiothreitol.

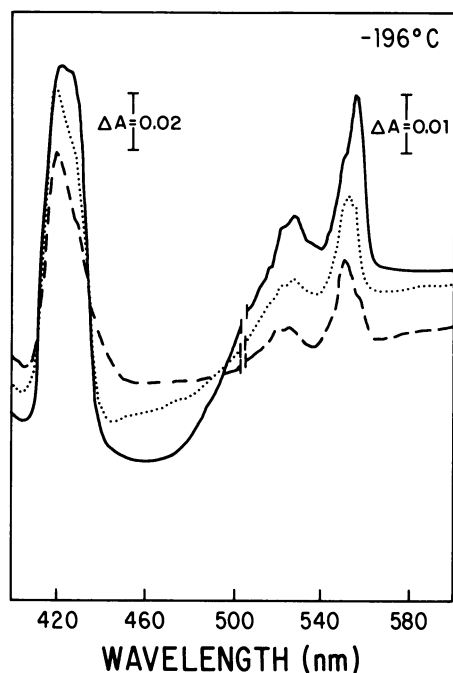


FIG. 2. Difference spectra (reduced versus oxidized) of membrane vesicles of *C. thermoautotrophicum* recorded at -196°C . The membrane vesicles were either not exposed (---), exposed to CO for 30 s at 50°C (—), or incubated with CO for 30 s, followed by exposure to CO_2 for 120 s at 50°C (·····). The reference was oxidized with air before being frozen. The reactions were done with 2.8 mg of protein per ml in 50 mM potassium phosphate (pH 7) containing 3 mM dithiothreitol.

to methyl- H_4 folate may allow energy generation (7, 20). This reduction is associated with a free energy change large enough to drive the phosphorylation of ADP. The fact that methylene- H_4 folate reductase is membrane bound agrees with this suggestion. The redox potential (E_0) for the methylene- H_4 folate/methyl- H_4 folate couple is -120 mV (2). Thus, methylene- H_4 folate can accept electrons from several electron carriers, e.g., ferredoxin, flavins, and cytochrome *b*, which are found in membranes of acetogenic bacteria (6, 8), and also from CO via the membrane-bound carbon monoxide dehydrogenase. These reactions, if coupled to ATP generation, can explain how *C. thermoautotrophicum* can grow on CO. Furthermore, they can supply energy in addition to that generated by substrate-level phosphorylation to heterotrophically growing cells, provided that CO is a product of decarboxylation of pyruvate.

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