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# Levels of Enzymes Involved in the Synthesis of Acetate from $CO_2$ in *Clostridium thermoautotrophicum*

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The acetogenic bacterium *Clostridium thermoautotrophicum*, grown on methanol, glucose, or $CO_2$-$H_2$, contained high levels of corrinoids, formate dehydrogenase, tetrahydrofolate enzymes, carbon monoxide dehydrogenase, and hydrogenase. Cell-free extracts catalyzed pyruvate-dependent formation of acetate from methyltetrahydrofolate. These results suggest that *C. thermoautotrophicum* synthesizes acetate from $CO_2$ via a formate-tetrahydrofolate-corrinoid pathway.

*Clostridium thermoaceticum*, *Clostridium formicoaceticum*, *Acetobacterium woodii*, *Clostridium aceticum*, and the newly isolated *Clostridium thermoautotrophicum* (27) ferment one hexose stoichiometrically to three acetates. The pathway which was recently reviewed (18) has been best elucidated with *C. thermoaceticum* (3, 28) and *C. formicoaceticum* (1, 19).

The Embden-Meyerhof pathway is utilized in the production of two acetates (1, 19, 28). In the production of the methyl group of the third acetate, $CO_2$ is first reduced to formate by formate dehydrogenase (2, 21, 26). Formate is further reduced to 5-methyltetrahydrofolate via a pathway with 10-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate, and 5,10-methylenetetrahydrofolate as intermediates. The levels of enzymes in this pathway are characteristically high in acetogens (3, 19, 25). The methyl group of methyltetrahydrofolate is probably transferred to a corrinoid enzyme. A recently isolated five-component system from *C. thermoaceticum* (11) carries out methyl transfer and condensation of this methyl group with carbon monoxide or the $C_1$ of pyruvate (23) to form acetate. Carbon monoxide dehydrogenase, which is present in *C. thermoaceticum* and *C. formicoaceticum* (8, 10), and a corrinoid enzyme are a part of the five-component system (11).

*A. woodii* (5, 22) and *C. thermoautotrophicum* (27) utilize molecular hydrogen and carbon dioxide as well as ferment hexoses in the synthesis of acetate. *A. woodii* cells grown on $H_2$-$CO_2$ have high levels of tetrahydrofolate pathway enzymes and corrinoids, and extracts from these cells carry out a pyruvate-dependent formation of acetate from methyl-$B_{12}$ and methyltetrahydrofolate (25). In this paper, we show that *C. thermoautotrophicum*, which forms acetate also from methanol (27) and carbon monoxide (J. Wiegel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I107, p. 112) has high levels of enzymes pertinent to acetate synthesis and corrinoids.

*C. thermoautotrophicum* (DSM 1974) was cultured as previously described (6) on methanol ($CO_2$ atmosphere), glucose ($CO_2$ atmosphere), or an $H_2$-$CO_2$ (66:33) gas mixture. To determine activities of 10-formyl-$H_4$folate synthetase (20, 24), 5,10-methenyl-$H_4$folate cyclohydrolase (17), and 5,10-methylene-$H_4$folate dehydrogenase (17), extracts were prepared with 2 g of wet cells. After suspending the cells in 8 ml of 50 mM potassium phosphate (pH 7), they were passed twice through a French pressure cell at 10,000 lb/in$^2$ and centrifuged at 5°C for 90 min at 100,000 × *g*.

To determine formate dehydrogenase, hydrogenase, and carbon monoxide dehydrogenase, extracts were prepared under anaerobic conditions as described by Ljungdahl and Andreesen (16). Oxygen was removed from $N_2$, $CO_2$, and $H_2$ by passage through a copper column heated to 175°C. Three grams of cells was suspended in 15 ml of 50 mM potassium phosphate (pH 6.8)–2 mM sodium dithionite–0.2 mM methyl viologen (MV)–0.1 mM phenylmethanesulfonyl fluoride–1 μg of DNase I per ml. After breaking the cells at 15,000 lb/in$^2$ in a $CO_2$-sparged French pressure cell, the suspension was centrifuged under an $N_2$-$H_2$ (95:5) atmosphere at 90,000 × *g* for 90 min at 5°C. All assays were performed at 60°C with the supernatants from the above centrifugations. Protein was determined by the method of Elliott and Brewer (12) with ovalbumin as the standard.

Hydrogenase and carbon monoxide dehydrogenase (carbon monoxide-MV oxidoreductase) activities were determined by following the reduction of MV at 600 mM with an extinction coefficient of 11.4 × 10$^3$ (7). The anaerobic assay mixture contained 30 mM MV, 3.2 mM dithio-

TABLE 1. Specific activities of *C. thermoautotrophicum* enzymes grown under different conditions

| Growth conditions | Sp act[a] | | | | | |
|---|---|---|---|---|---|---|
| | Formate dehydrogenase[b] | 10-Formyl $H_4$folate synthetase | 5,10-Methenyl $H_4$folate cyclohydrolase | 5,10-Methylene $H_4$folate dehydrogenase[c] | Carbon monoxide dehydrogenase[b] | Hydrogenase[b] |
| $H_2 + CO_2$ | 0.036 | 10.8 | 0.49 | 2.03 | 10.7 | 0.31 |
| Glucose + $CO_2$ | 0.072 | 11.1 | 0.63 | 0.82 | 3.8 | 0.16 |
| Methanol + $CO_2$ | 0.070 | 7.7 | 0.94 | 3.10 | 7.8 | 0.08 |

[a] Specific activity is defined as micromoles of substrate converted or product formed per minute per milligram of protein.
[b] MV was used as the electron acceptor.
[c] NADP, but not NAD, served as the electron acceptor.

threitol, and 100 mM potassium phosphate. Oxygen-free hydrogen gas or carbon monoxide (99.99%; Airco Industrial Gases) was bubbled into the assay mix in a serum-stoppered cuvette at 60°C for 5 min before enzyme was added to start the reaction. The nonspecific reduction of the viologen dye was corrected for by using nitrogen as the gas phase. The activity of formate dehydrogenase was determined with MV as the electron acceptor (16). The pyruvate-dependent conversion of [*methyl*-$^{14}$C]methyltetrahydrofolate to acetate was performed as described by Ghambeer et al. (13). The extraction of total corrinoids was performed by the method of Bernhauer et al. (4). The corrinoids as dicyanocorrinoids were determined spectrophotometrically at 368 and 580 nm with the extinction coefficients $30.8 \times 10^3$ and $10.6 \times 10^3$ $M^{-1}$ $cm^{-1}$, respectively (14).

TABLE 2. Incorporation of the *methyl*-$^{14}$C moiety of [*methyl*-$^{14}$C]methyl-$H_4$folate into acetate by cell-free extracts of *C. thermoautotrophicum* grown under different conditions[a]

| Growth conditions | Protein in assay (mg) | Reaction system | Acetate formed (dpm) | % Conversion ± SD |
|---|---|---|---|---|
| $H_2$ + $CO_2$ | 11.3 | Complete mix | 196,500 | 25 ± 2 |
| | | Minus coenzyme A | 260,100 | 33 ± 4 |
| | | Minus pyruvate | 10,580 | 1 ± 0.6 |
| Glucose + $CO_2$ | 5.5 | Complete mix | 277,400 | 35 ± 3 |
| Methanol + $CO_2$ | 9.1 | Complete mix | 57,100 | 7 ± 1 |

[a] The complete incubation mixture contained, in 1 ml, sodium pyruvate (30 μmol), dithiothreitol (10 μmol), ferrous ammonium sulfate (5 μmol), coenzyme A (3.3 μmol), (±)-[*methyl*-$^{14}$C]methyl-$H_4$folate (0.85 μmol, 796,800 dpm), potassium phosphate (pH 7.0; 50 μmol), and 400 μl of each crude extract. The incubation was for 10 min at 60°C under $N_2$.

The levels of the tetrahydrofolate enzymes, formate dehydrogenase, hydrogenase, and carbon monoxide dehydrogenase are shown in Table 1. The 10-formyltetrahydrofolate synthetase, 5,10-methenyltetrahydrofolate cyclohydrolase, and 5,10-methylenetetrahydrofolate dehydrogenase activities were similar to those in *C. thermoaceticum*, *C. formicoaceticum*, and *A. woodii* (3, 19, 25). The methylenetetrahydrofolate dehydrogenase was exclusively NADP dependent, as in *C. thermoaceticum* (17).

The activity of formate dehydrogenase in *C. thermoautotrophicum* is lower than in *C. formicoaceticum* (15) and *C. thermoaceticum* (2) when MV is used as the electron acceptor. In *C. thermoaceticum*, formate dehydrogenase reacts with NADP at a rate of 50% that with MV, and NADP is considered to be the natural electron acceptor (2, 21, 26). Although a low activity with NAD has been found with formate dehydrogenase from *C. formicoaceticum*, the natural electron carrier has yet to be found (15). Similarly, a weak formate-dependent reduction of NADP was observed with extracts of *C. thermoautotrophicum*, but it was only about 4% that with MV, and we do not believe that NADP is the natural carrier.

Carbon monoxide dehydrogenase activity was high in extracts of *C. thermoautotrophicum*. In the glucose-grown cells, the activity was one-third of that in the $H_2$-$CO_2$-grown cells. We found the carbon monoxide dehydrogenase activity in *C. thermoaceticum* and *A. woodii* to be comparable to that in *C. thermoautotrophicum*.

The highest activity of hydrogenase was found in $H_2$-$CO_2$-grown cells of *C. thermoautotrophicum*; the glucose- and the methanol-grown cells had 50 and 25%, respectively, of that of the $H_2$-$CO_2$-grown cells. Similarly, in autotrophically grown cells of *C. aceticum*, the hydrogenase activity is much higher than in heterotrophically produced cells (5). Hydrogenase has recently been found in *C. thermoaceticum* (9).

Between 50 and 70% of the available racemic mixture of [*methyl*-$^{14}$C]methyltetrahydrofolate

was incorporated into acetate in the presence of pyruvate with extracts from cells grown on $H_2$-$CO_2$ and glucose (Table 2). A lower value (14%) was obtained with the methanol-grown cells. The levels of corrinoids in extracts of *C. thermoautotrophicum* grown on $H_2$-$CO_2$, glucose, or methanol were, respectively, 3.0, 1.8, and 2.7 μmol/g of soluble protein.

The results presented indicate that, in *C. thermoautotrophicum*, acetate is produced from $CO_2$-$H_2$ and from glucose-generated $CO_2$ and electrons by a pathway involving formate, tetrahydrofolate derivatives, and corrinoids, similarly to other acetogenic bacteria (18). The high levels of the tetrahydrofolate enzymes found in methanol-grown cells also indicate a role for these enzymes with this substrate.

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