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One Carbon Metabolism in Anaerobic Bacteria: Regulation of Carbon and Electron Flow During Organic Acid Production

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SUMMARY

Our project deals with understanding the fundamental biochemical mechanisms that physiologically control and regulate carbon and electron flow in anaerobic chemosynthetic bacteria that couple metabolism of single carbon compounds and hydrogen to the production of organic acids (formic, acetic, butyric, and succinic) or methane. We compare the regulation of carbon dioxide and hydrogen metabolism by fermentation, enzyme, and electron carrier analysis using *Butyribacterium methylotrophicum*, *Anaerobiospirillum succiniciproducens*, *Methanosarcina barkeri*, and a newly isolated tri-culture comprosed of a syntrophic butyrate degrader strain IB, *Methanosarcina mazei* and *Methanobacterium formicicum* as model systems. To understand the regulation of hydrogen metabolism during butyrate production or acetate degradation, hydrogenase activity in *B. methylotrophicum* or *M. barkeri* is measured in relation to growth substrate and pH; hydrogenase is purified and characterized to investigate number of hydrogenases; their localization and functions; and, their sequences are determined. To understand the mechanism for catabolic CO_2 fixation to succinate the PEP carboxykinase enzyme and gene of *A. succiniciproducens* are purified and characterized. Genetically engineered strains of *Escherichia coli* containing the phosphoenolpyruvate (PEP) carboxykinase gene are examined for their ability to produce succinate in high yield. To understand the mechanism of fatty acid degradation by syntrophic acetogens during mixed culture methanogenesis formate and hydrogen production are characterized by radiotracer studies. It is intended that these studies provide strategies to improve anaerobic fermentations used for the production of organic acids or methane and, new basic understanding on catabolic CO_2 fixation mechanisms and on the function of hydrogenase in anaerobic bacteria.

SCIENTIFIC BACKGROUND

One carbon and H_2 metabolism is associated with the degradation and synthesis of organic compounds during fermentations that provide energy for diverse species of anaerobic bacteria (Thauer et al., 1977, Zeikus, 1980). Methanogenic and acetogenic bacteria derive energy as a consequence of linking H_2 consumption with CO_2 reduction into methane or acetic acid. In these two different metabolic systems the functions of hydrogenase and the key CO_2 fixing enzymes for both catabolism and anabolism are shared and have been described (see Bhatnagar et al., 1991; Wood & Ljungdahl, 1991 for recent reviews).

Both methanogens and acetogens employ a carbonyl dependent acetyl CoA synthesis pathway of anabolism where $H_2 + CO_2$ are reduced to CH_3B_{12} and CO which are condensed via CO dehydrogenase into acetyl CoA (Zeikus et al., 1985). Acetogens also use the carbonyl dependent acetyl CoA pathway (or Wood pathway) for catabolism (Wood et al., 1986). Dependent on the acetogenic species acetyl CoA can be converted to either acetic acid, butyric acid, ethanol or butanol. The acetogen we study, *Butyribacterium methylotrophicum* produces spores and ferments CO or glucose into all of these products (Lynd et al., 1982; Kerby et al., 1983, Lynd & Zeikus, 1983: Worden et al., 1989 see progress report).

Methanosarcina barkeri is one of the most catabolically diverse methanogen and it ferments H_2/CO_2, acetate, methanol, CO and methylamines. H_2 production and consumption is associated with both acetate and CO metabolism in *M. barkeri* (Krzycki et al., 1987; O'Brien et al., 1984; Bhatnagar et al. 1991). Most of the methane formed in nature (~70%) is derived from acetate fermentation. Methanogenesis from acetate proceeds by cleavage of acetyl CoA via CO dehydrogenase (Krzycki & Zeikus, 1984a) into a methyl moiety and a carbonyl moiety which serve as the electron acceptor and electron donor for methane and CO_2 production. The role of hydrogen and the mechanism of electron transfer between the C_1 and C_2 of acetate is the focus of the present research. We have proposed that this transfer is mediated by membrane bound electron carriers and enzyme with concommittant translocation of protons and reconsumption of the gradient to form ATP (Krzycki & Zeikus, 1984a; Krzycki et al., 1985; Kemner et al., 1987; Peinemann et al., 1988) have shown that methanogenesis from acetate leads to the formation of a transmembrane proton motive force, which can drive ATP synthesis. Cytoplasmic hydrogenase have been purified from *M. barkeri* strains which reduce F_{420} (Fiebig & Feidrich, 1989; Fauque et al., 1984) and a ferredoxin (Hausinger et al., 1982) have been purified. Kemner et al. (1987) demonstrated that cytochrome b in *M. barkeri* membranes was reduced by hydrogenase and oxidized by methyl CoM reductase activities.

Anaerobic bacteria can also conserve energy as a consequence of fixing CO_2 into succinate but this biochemistry has not been well characterized (Thauer et al., 1977; Zeikus, 1980). Understanding of the biochemistry of succinate fermentations have focused on characterization of fumarate reductase and

electron transport coupled phosphorylation during growth of *W. succinogenes* grown on H_2 or formate plus fumarate (Kroger, 1980). Recently, succinate efflux has been shown to be linked to transport coupled phosphorylation in *Bacteroides* (Michel & Macy, 1990).

The catabolic pathway for glucose fermentation in *A. succiniciproducens* was propsed by Samuelov et al. (1991). It has been shown that the presence of CO_2 as an electron acceptor switches glucose catabolism from a lactate-ethanol fermentative process to a succinate respirative process. Phosphoenol (PEP) carboxykinase was the key regulated enzyme in the pathway leading to succinate production, and its functions as a catabolic CO_2 fixing enzyme. By and large previous studies on PEP carboxykinase deal with the anabolic function of this enzyme. It is recognized as being a key enzyme in gluconeogenesis in different organisms from bacteria to vertebrates (Utter & Kolenbrander, 1972). Unlike these enzymes, PEP carboxykinase from *A. succiniciproducens* plays an important role in catabolic CO_2 fixation. The PEP carboxykinase of *A. succiniciproducens* was purified and characterized (Podkovyrov & Zeikus, 1992). The enzyme was monomeric with an M_r of 57 000 $\pm$ 2 000. The enzyme was ADP-dependent, oxygen stable, had a pH optimum of 6.5 - 7.1, and was stable from pH 5.0 to 9.0. The N-terminal sequence of the enzyme showed the most similarity to the enzyme from *Escherichia coli*. There are three genes encoding for ATP-dependent PEP carboxykinases sequenced to date from *E. coli, Trypansoma brucei* and *Saccharomyces cerevisiae* (Medina et al., 1990). All of these enzymes function in vivo catalyzing formation of PEP from oxaloacetate.

We recently discovered that HCO_3^- is fixed into formate during syntrophic degradation of ethanol or butyrate (Thiele & Zeikus, 1988a,b; see progress report). This result was unexpected as synthrophic acetogenic bacteria which degrade ethanol or butyrate were thought to be obligate proton reducing bacteria that only produced H_2 and acetic acid which are simultaneously degraded by methanogenic partners (McInerney, 1988; Schink, 1992). This metabolic coupling between the syntrophiles and the methanogens is called interspecies H_2 transfer and is well documented (Zeikus, 1983; Schink, 1992). Interspecies formate transfer as a consequence of bicarbonate reduction by syntrophiles is more thermodynamically favorable (see progress report) than H^+ reduction to H_2 but it remains to be better documented. Bicarbonate is reduced to formate by anaerobes either by formate dehydrogenase or CO_2 reductase (Thauer et al., 1977).

Hydrogenases have been characterized in diverse anaerobic bacteria that consume H_2 as an energy source or that produce H_2 as an end product of organic fermentations (see Przybyla et al., 1992 for a recent review). It is worth noting that we know a lot about the biochemistry of hydrogenase and we are learning more about hydrogenase genetics. However, we know almost nothing about the regulation of hydrogenase and its physiological function in anaerobes that produce and consume hydrogen during the fermentation of organic matter. The

production and consumption of H_2 during growth of anaerobes on organic substrates has been demonstrated during sulfidogenic fermentations (Odom & Peck, 1981), solventogenic fermentation (Kim et al., 1984), methogenic fermentation (Krzycki et al., 1987; O'Brien et al., 1984) and recently during carbonyl dependent acetogenic fermentations (see progress report). We intend to demonstrate how hydrogen production and consumption during acetate degradation by *M. barkeri* and during acetate synthesis in *B. methylotrophicum* is regulated by multiple hydrogenases and related to energy conservation (see progress report and proposed research).

Many bacteria produce multiple hydrogenases whose functions are not known (Adams et al., 1981). Hydrogenases also differ widely in regard to molecular composition, electron carrier specificity, kinetic properties, metal content, cellular localization etc. To date, hydrogenases have been purified, cloned, sequenced and characterized from diverse aerobic and anaerobic H_2 consuming or producing bacteria (Przybyla et al., 1992). The hydrogenase genes of methanogens but not acetogens have been characterized (Reeve & Beckler, 1990; Bhatnagar et al., 1991; Wood & Ljungdahl, 1991).

SIGNIFICANCE

The proposed research will provide new understanding on the regulation of carbon and electron flow pathways in anaerobic bacteria that employ one carbon metabolism in the synthesis or degradation of organic acids.

The biochemical properties and function of membrane bound hydrogenase associated with acetate degradation to methane by *M. barkeri* will be characterized. The biochemical properties and function of hydrogenases associated with the carbonyl dependent acetogensis pathway of glucose degradation by *B. methylotrophicum* will be characterized. In addition, the hydrogenase genes of this acetogen will be cloned and compared to those of sulfate reducers, methogens and fermentative bacteria described. This work will provide new physiological understanding on hydrogenase regulation and function in controlling carbon and electron flow in response to changes in fermentation substrates or end products.

The biochemical properties and physiological functions of two understudied catabolic CO_2 fixing reactions - PEP carboxykinase and CO_2 reductase will be elucidated. The former enzyme is the key CO_2 fixing enzyme for catabolic succinate production and the latter enzyme may be very important in the anaerobic degradation of fatty acids by mixed cultures of syntophiles and methanogens.

The proposed research will also provide strategies for utilizing and controlling anaerobic bacterial fermentations used in the production of energy

intensive chemicals, and in waste treatment systems. Anaerobic bacterial fermentations have application in the direct fermentation of plant biomass substrates (starch, cellulose, glucose) into high value organic acids (lactic, succinic, butyric, acetic) and organic solvents (i.e., butanol, acetone and ethanol), or in the fermentation of synthesis gas ($CO + H_2$) or methanol derived from pyrolysis of coals and biomass residues into CH_4, or oxychemicals (Zeikus, 1980). Most glucose based fermentations and the *B. methylotrophicum* CO fermentation produce multiple end products. Knowledge on how enzymes control carbon and electron flow in these fermentations is a key in understanding how to over produce a particular valuable end product as a consequence of elimination of a waste product. For example, just controlling production hydrogenase activity by inhibition with CO in the glucose fermentation by *Clostridium acetobutylicum* increased the butanol yield by 30% as a consequence of increasing butyric acid consumption and reduction (Kim et al., 1984). The current research should yield similar strategies for improving butyric acid and succinic acid based fermentation systems.

Succinic acid fermentations are particularly important for conversion of plant biomass into energy intensive chemicals and fuels. Succinic acid can be hydrogenated to 1-4 butanediol, the precursor to stronger than steel plastics used in manufacturing; and succinic acid is preferred over maleic anhydride, the current chemical feedstock used for 1-4 butanediol production (Jain et al., 1989). The succinate fermentation can be improved as the following reaction is feasible in theory (i.e., provided the organism has the right enzymes)

$$1\text{Glucose} + 2CO_2 + 1H_2 \rightarrow 2\ \text{Succinate}$$

The succinate fermentation should be of interest to corn processors because industrial ethanol production from starch derived glucose fermentations produce copious amounts of CO_2 as waste end product. The uses for this waste CO_2 could be as the electron acceptor for a glucose based succinate fermentation process. We can predict that cloning the catabolic PEP carboxykinase gene into *E. coli* could convert it to a higher yielding succinate strain. This is in analogy to the work of Ingraham and coworkers who cloned the pyruvate decarboxylase and alcohol dehydrogenase genes of *Zymomonas mobilis* into *E. coli* and made the recombinant E. coli strain suitable for producing ethanol from hexose and pentose sugars.

Lastly, knowledge on the mechanism of degradation of acetate and butyrate and the role of hydrogen and CO_2 metabolism in these processes is crucial to understanding the anaerobic digestion of waste into methane. Butyrate degradation is often the rate limiting step in overall organic decomposition of soluble wastes and acetate degradation is rate limiting for methanogenesis. To improve anaerobic digestion systems, we need to understand the exact metabolites formed so as to develop monitoring systems based on them for improved process control.

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