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IN VITRO METHANE AND METHYL COENZYME M FORMATION FROM ACETATE: EVIDENCE THAT ACETYL-CoA IS THE REQUIRED INTERMEDIATE ACTIVATED FORM OF ACETATE

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SUMMARY: Buffer-soluble extracts of acetate-grown Methanosarcina barkeri catalyzed methanogenesis from acetate in the presence of hydrogen and ATP. The rates of methane formation from either acetate plus ATP, or acetylphosphate without ATP added, were approximately doubled by the addition of coenzyme A (CoA). In vitro methyl group transfer from [2-^{14}C]acetate to form [$^{14}CH_3$]methyl coenzyme M ($^{14}CH_3S$-CoM) was monitored by causing the accumulation of $^{14}CH_3S$-CoM ($^{14}CH_3$-$SCH_2CH_2SO_3^-$) in the presence of 2-bromoethanesulfonate. The rate of $^{14}CH_3S$-CoM formation was increased 2.5-fold by 0.2 mM CoA.

A system developed for assay of methanogenesis from acetate by buffer-soluble extracts of Methanosarcina barkeri was described in the reports of Krzycki et al. (1,2). Conversion of acetate to methane and carbon dioxide was carried out under an atmosphere of hydrogen and was dependent upon ATP. With acetylphosphate as substrate ATP was not required (2). It has been suggested that activation of acetate would be necessary for carbon-carbon bond cleavage to proceed (3), and the requisite high levels of acetate kinase and phosphotransacetylase are present in extracts of M. barkeri (4,5). However, direct experimental evidence for acetate activation in methanogenesis is lacking, and the identity of the activated form is unknown. Moreover, there have been no subsequent reports to confirm and extend the studies on in vitro methanogenesis from acetate. In the present report we describe the stimulatory effects of coenzyme A (CoA) addition on the formation of methyl coenzyme M (CH_3S-CoM) in an in vitro assay system and on the overall conversion of acetate to methane by buffer-soluble extracts of acetate-grown M. barkeri. The results of these studies indicate an important role of acetyl-CoA in the pathway of methanogenesis from acetate.

MATERIALS AND METHODS

Culture of M. barkeri on acetate was carried out as described previously (6). Buffer-soluble cell extracts were prepared within the National Institutes of Health Anaerobic Laboratory (7). The cells were washed with hydrogen-sparged

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50 mM potassium 3-(N-morpholino)propanesulfonate buffer (MOPS), pH 7.0, and disrupted by twice passage through a French pressure cell. Following incubation with DNAase the cellular debris was removed by centrifugation for 20 min at 16,000 x g. The supernatant (approx. 3 ml per g wet-packed cells) was immediately frozen by dripping into liquid nitrogen and stored at -70°C. Frozen pellets were thawed under H_2 just prior to use. Protein measurements were performed by the method of Bradford (8) using the reagent purchased from Bio-Rad Laboratories. All other experimental details are provided in the appropriate figure legends.

RESULTS AND DISCUSSION

In vitro assays of methanogenesis in the presence and absence of acetate are shown in Figure 1. Methane production was dependent upon hydrogen and ATP as described originally by Krzycki et al. (1,2). Without addition of acetate a small amount of methane was formed at a low rate from endogenous substrates. With acetate as substrate methanogenesis was rapid and prolonged, and addition of 0.1 mM CoA doubled the maximal observed rate. The total methane produced increased to 2.6 times that found in the absence of CoA. Similarly, in experiments where acetylphosphate was substituted for acetate plus ATP (not shown), the maximal rate of methane formation in the presence of 0.2 mM CoA (or 0.2 mM acetyl-CoA) was twice that observed in reactions wherein CoA was omitted. These results suggest that acetyl-CoA rather than acetylphosphate is the intermediate form of acetate which may undergo carbon-carbon bond cleavage. The lag noted

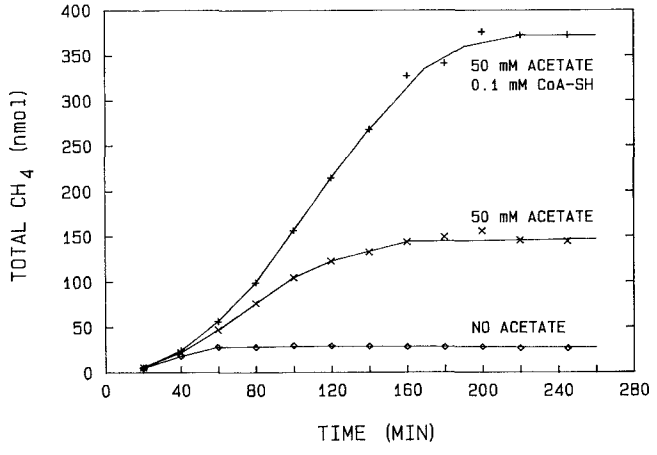

Figure 1. The effect of CoA on in vitro methane formation from acetate. Methane production was measured by gas chromatography of 0.20 cc aliquots removed at the indicated times from the gas phase (8.0 cc) of vials containing a crude, buffer-soluble extract from acetate-grown M. barkeri prepared as described in "Materials and Methods". The reactions were buffered at pH 7.0 with 40 mM MOPS and contained 2.0 mg of crude extract protein, ATP (12.5 µmol), and $MgCl_2$ (6.0 µmol) in a final volume of 250 µl. Additions of sodium acetate (12.5 µmol) and sodium acetate plus CoA (0.025 µmol) are indicated. The vials were pressurized to 16 psi (approx. 110 kPa) with hydrogen and incubated at 37°C. The calculated total methane formed per vial uncorrected for aliquot removal is plotted versus reaction time.

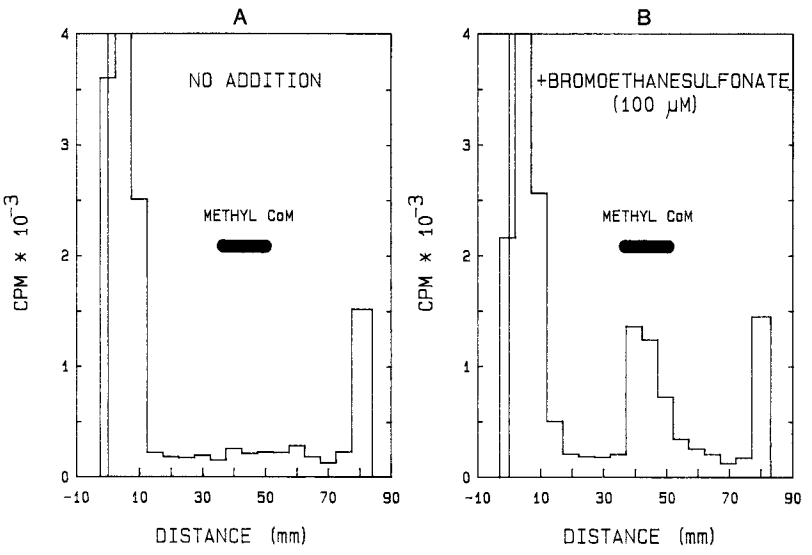

Figure 2. Thin-layer chromatographic analysis of products formed upon incubation of a crude, cell-free extract of acetate-grown M. barkeri with [2-^{14}C]acetate in the presence and absence of sodium 2-bromoethanesulfonate. Reactions at 37 °C were carried out with hydrogen as the gas phase (approx. 110 kPa overpressure) in a total volume of 204 µl containing the crude extract, 8 µmol of sodium acetate (4 x 10^6 cpm), 4.76 µmol ATP, and 40 nmol of sodium 2-thioethanesulfonate (CoM) either in the presence of 20 nmol of sodium 2-bromoethanesulfonate (A) or in its absence (B). The reactions were stopped after 125 min by addition of 0.8 ml of ethanol. Carrier methyl-CoM (0.5 µmol) was introduced and, after centrifugation, 100 µl of acetic acid was mixed with the supernatant. This was dried in vacuo. The residue was redissolved and dried twice more with 1.0 ml portions of 10% acetic acid in ethanol. A small amount of precipitate was removed after final dissolution in water and 10 µl (one half of the total volume) was streaked on an Eastman Kodak 13255 cellulose TLC plate cut from a 20 x 20 cm sheet. The chromatogram was developed with a solvent consisting of acetone:ammonium hydroxide:water (16:1.8:10). The location of methyl-CoM (indicated by the bar) was determined by spraying with a chloroplatinic acid/KI reagent, and 0.5 cm sections were scraped from the plate and analyzed for radioactivity.

in methane formation (Fig. 1) is probably due to activation of CH_3S-CoM methylreductase since methanogenesis from CH_3S-CoM plus ATP (data not shown) exhibited a similar induction period. Furthermore (see later), no lag is apparent in CH_3S-CoM formation from acetate.

The involvement of CH_3S-CoM methylreductase in methanogenesis from acetate was suggested earlier (2) by the finding that 2-bromoethanesulfonate, a powerful inhibitor of CH_3S-CoM methylreductase (9), blocked methane formation from acetate. Detection of $^{14}CH_3S$-CoM formation from [2-^{14}C]acetate during in vitro methanogenesis from acetate was taken as evidence that CH_3S-CoM is an intermediate in the pathway (2). The results shown in Figure 2A indicate that under these conditions very little $^{14}CH_3S$-CoM accumulated during in vitro methanogenesis from [2-^{14}C]-acetate. However, when 2-bromoethanesulfonate was added to inhibit CH_3S-CoM reduction, a substantial amount of $^{14}CH_3S$-CoM accumulated

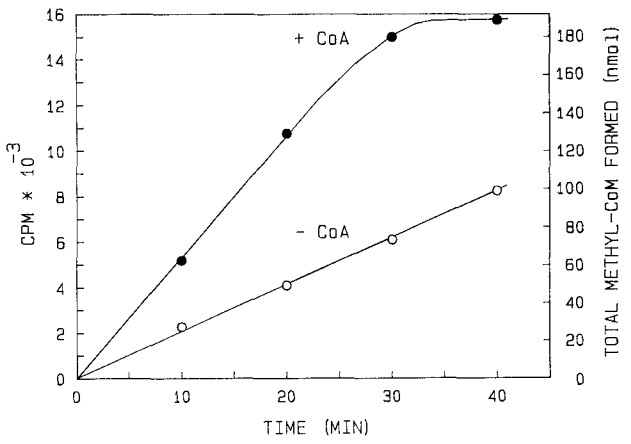

Figure 3. Time course of methyl-CoM formation from acetate in the presence and absence of CoA by a crude, buffer-soluble extract of acetate-grown M. barkeri. At the indicated times 50 µl aliquots were removed from reactions carried out under conditions analogous to those given in Fig. 2B, except that the total volume was 300 µl and contained 4.14 mg of protein, 6.9 µmol ATP, 12 µmol sodium acetate (6 x 10^6 cpm), 50 nmol sodium 2-bromoethanesulfonate, 6 µmol $MgCl_2$, 240 nmol CoM, and where indicated, 60 nmol CoA. The aliquots were added immediately to 450 µL of 86% ethanol and prepared for thin-layer chromatography in a manner similar to that given in Fig. 2. The position of methyl-CoM was located on the plate as described in Fig. 2, and a 17 mm zone containing the compound was scraped and analyzed for radioactivity. In this experiment the crude extract was prepared from a different batch of cells, and exhibited a substantially higher activity than the extract used in Fig. 2.

(Figure 2B). It was found that hydrogen was required, and we speculate that this may be due to reductive activation of a corrinoid methyl group carrier by analogy with methanol: 5-hydroxybenzimidazolylcobamide methyltransferase activation (10). These findings lend further support to the conclusions of Krzycki et al. (2) regarding the intermediacy of CH_3S-CoM and the involvement of CH_3S-CoM methylreductase in methanogenesis from acetate.

To investigate the effects of CoA on CH_3S-CoM formation from acetate we have used the method described in Figure 2B. The time course of $^{14}CH_3S$-CoM formation from [2-^{14}C]acetate is shown in Figure 3. Marked stimulation of $^{14}CH_3S$-CoM formation was observed upon addition of reduced CoA (upper curve). The rate of CH_3S-CoM production from acetate was 2.5 times greater in the presence of 0.2 mM CoA than in the absence of added CoA. Since the initial rates of $^{14}CH_3S$-CoM production were identical with either 0.2 mM or 0.8 mM levels of acceptor 2-thioethanesulfonate (data not shown), this indicates that the enzyme system is saturated at these levels and thus addition of free sulfhydryl groups per se would not account for the enhancement of rate observed with reduced CoA. These results support the view that acetyl-CoA, a likely substrate for carbon-carbon bond cleavage, is an important intermediate in the pathway of methanogenesis from acetate.

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