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Importance of Tetrahydrofolate and ATP in the Anaerobic O-Demethylation Reaction for Phenylmethylethers

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DL-Tetrahydrofolate (THF) and ATP were necessary for the anaerobic O-demethylation of phenylmethylethers in cell extracts of the type strain (ATCC 29683) of the homoacetogen *Acetobacterium woodii*. The reactants for this enzymatic activity have not been previously demonstrated in any system, nor has the mediating enzyme been studied. An assay using reaction mixtures containing 1 mM THF, 2 mM ATP, and 2 mM hydroferulate (i.e., 4-hydroxy,3-methoxyphenylpropionate) was developed and was performed under stringent anaerobic conditions. Pyridine nucleotides and several other possible cofactors were tested but had no effect on the activity. After centrifugation of disrupted cells at 27,000 × *g*, the activity was found primarily in the supernatant, which had a specific activity of 14.2 ± 0.5 nmol/min/mg of protein. At saturating levels of each of the other two substrates, apparent K_m values for the variable substrate were 0.65 mM hydroferulate, 0.27 mM ATP, and 0.17 mM THF. Activity was significantly decreased when extract was preincubated at 60°C and was completely lost after preincubation in air for 30 min. Thus, the soluble anaerobic O-demethylating enzyme system of *A. woodii* is oxygen sensitive. The THF- and ATP-dependent activity measurable in the soluble fraction of cell extracts constituted about 34% of the activity seen with intact cells.

Anaerobic O-demethylation of phenylmethylethers is apparently an important process in anaerobic environments, yet very little is known about the mechanism of this reaction. Aryl-*O*-methyl ethers are abundant in natural products, including plant phenolics, particularly as components of lignin structural polymers (29). Methoxylated lignin monomers can be completely degraded under anaerobic conditions by methanogenic microbial enrichments (11) and by defined cocultures (26). Anaerobic O-demethylation is an early step in this process. It is also a key reaction in the utilization of the *O*-methyl substituent as a one-carbon substrate by acetogens, i.e., bacteria that synthesize acetate from one-carbon compounds or precursors (27).

Bache and Pfennig (3) first discovered isolates which grew, in the presence of carbon dioxide, on the methoxyl carbon of aryl-*O*-methyl compounds as a one-carbon substrate. Their isolates, originating from sludge and freshwater sediments, were recognized as homoacetogens and classified as *Acetobacterium woodii* (4). Strains capable of anaerobic O-demethylation have been isolated subsequently from a variety of anaerobic habitats such as the gut, the rumen, sediments, and sewage sludge and include both mesophiles and thermophiles (13, 37). With the exception of the acetogens, the capacity for anaerobic O-demethylation is not very common among bacteria, although bacteria with this capability belong to diverse physiological groups. However, it is recognized that most acetogens can mediate this key reaction. Acetogens are not capable of cleaving the aromatic ring, even though they may metabolize several types of ring substituents in addition to *O*-methyl groups (3, 22, 28).

The biochemical mechanism for anaerobic O-demethylation is not yet known, nor have the reactants for this activity been identified. Consequently, the way in which the carbon flow pathway for *O*-methyl substituents might connect to the central Wood pathway for autotrophic or one-carbon acetyl coenzyme A (acetyl-CoA) synthesis in acetogens (27, 35) remains to be established. Although similarities between the one-carbon metabolism of *O*-methyl substituents and that of methanol would be anticipated, several lines of evidence suggest that the initial metabolic reactions are distinct. Methanol seems not to be an intermediate during growth of *A. woodii* on methoxylated compounds (34). Cells of the acetogen *Eubacterium limosum* that are adapted for growth on methanol require de novo protein synthesis to become induced for O-demethylation and exhibit a lag when transferred to growth media containing methoxylated compounds (14). The protein profile of *Clostridium thermoaceticum* grown on methoxylated aromatics is distinct from that of methanol-grown cells (13). In growth studies of the metabolism of 3-[methoxy-^{18}O]benzoate by *A. woodii* and *E. limosum*, DeWeerd et al. (14) showed that the ^{18}O label is retained in the 3-hydroxybenzoate product after O-demethylation. Thus, the ether linkage is cleaved between the methyl group and the aryl oxygen, suggesting that the anaerobic O-demethylation reaction involves methyl group transfer.

Previous attempts to study the O-demethylating activity in cell extracts have met with limited success. The level of activity has been either rather low (16, 19) or sensitive to fractionation (12, 36). The present work is directed toward describing the reactants for anaerobic O-demethylation. It is also the first report of successful conditions for the assay of this activity in the cytoplasmic fraction of cell extracts.

MATERIALS AND METHODS

Bacterial strain and procedures. The type strain of *A. woodii*, ATCC 29683, was obtained from the American Type Culture Collection. Cells to be used in enzyme assays were grown in 6-liter batches at 30°C under N_2-CO_2 (70:30) in a defined medium modified from that of Frazer and Young

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(17). This medium contained the following (in grams per liter): $NaHCO_3$, 5.0; L-cysteine, 0.35; NH_4Cl, 0.2; $MgCl_2 \cdot 6H_2O$, 0.18; and KCl, 1.3. Additional ingredients (in milligrams per liter) were the following: $(NH_4)_2PO_4$, 40; $FeCl_2 \cdot 4H_2O$, 1.8; $MnCl_2 \cdot 4H_2O$, 20; $CoCl_2 \cdot 6H_2O$, 30; H_3BO_3, 5.7; $CaCl_2 \cdot 2H_2O$, 2.7; $Na_2MoO_4 \cdot 2H_2O$, 2.5; $ZnCl_2$, 2.1; resazurin, 1.0; $NiCl_2 \cdot 6H_2O$, 0.05; nitrilotriacetic acid, 12.8; $Na_2SeO_3 \cdot 5H_2O$, 0.003; and $Na_2WO_4 \cdot 2H_2O$, 0.004. A mixture of vitamins was added as previously described, and the carbon source was 0.1% fructose. Stock cultures were maintained by monthly transfers in the semidefined medium of Balch et al. (4), which contains a small amount of yeast extract prepared as described for medium 1019 (1).

When the culture reached the late exponential phase, as judged by a turbidity of 0.6 optical density units at 600 nm, 2 mM ferulate (i.e., 3-methoxy,4-hydroxycinnamate) was added to induce anaerobic O-demethylating activity in cells. After 2 to 3 h of induction, i.e., when about 75% of the ferulate had been converted to hydroferulate or hydrocaffeate, cells were harvested anaerobically at 4°C by centrifugation at 11,500 × *g* for 20 min in a JA-10 rotor of a Beckman (San Ramon, Calif.) J-21C centrifuge. The cell pellet was resuspended in a volume of anaerobic 30% glycerol solution (prepared in deoxygenated deionized water containing 0.025% cysteine) that was approximately equal to the volume of the packed cell pellet. The cell slurry, which contained about 1 g (wet weight) of cells ml^{-1}, was stored frozen at −20°C until disrupted.

Sources and preparation of biochemical compounds. Unless otherwise stated, the substrate used in reaction mixtures for the assay of anaerobic O-demethylating activity was hydroferulate, which was prepared from ferulate as a courtesy in the laboratory of Stephen Wilson, Department of Chemistry, New York University, by a catalytic hydrogenation method similar to the procedure of Chesson et al. (10). Other chemicals and biochemicals were obtained from various vendors and were of the highest purity available.

DL-Tetrahydrofolate (THF) was prepared in two steps involving reduction of folate to dihydrofolate by dithionite and subsequent further reduction to THF by sodium borohydride (32). THF was separated from residual unreacted folate or dihydrofolate in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under an atmosphere of N_2-H_2 (97:3) by adsorption to a quaternary amine anion-exchange solid-phase extraction column (Applied Separations, Allentown, Pa.) and elution with 0.2 M Tris (pH 7.2) buffer containing 5 mM dithioerythritol as described by Schieffer et al. (31). This preparation of THF in 0.2 M Tris–5 mM dithioerythritol was added directly to reaction mixtures for the assay of enzyme activity and was stored under an argon atmosphere at −20°C until use. The THF was quantitated by heating with concentrated formic acid to convert THF to methenyltetrahydrofolate, which was assayed by A_{355} (32).

The corrinoid cob(II)alamin (B_{12r}) was prepared from cyanocobalamin (i.e., vitamin B_{12} [Sigma Chemical Co., St. Louis, Mo.]) by reduction with dithioerythritol at 60°C in a modification of the method of Ankel-Fuchs and Thauer (2). A solution of 0.1 mM cyanocobalamin in 50 mM potassium phosphate buffer, pH 6.6, with 50 mM dithioerythritol was incubated for 3 h in an amber vial under argon at 60°C. Formation of the reduced cob(II)alamin product was confirmed by UV-visible spectroscopy.

Cell disruption and handling of extracts. Cells were disrupted inside the anaerobic chamber with a Mini-beadbeater (Biospec Products, Bartlesville, Okla.) with 0.1-mm zirconium beads that had been pretreated as recommended by the manufacturer. The cell slurry was routinely amended with 3 mM pyruvate, 0.05 mM ferulate, and 8 mM $MgCl_2$ prior to disruption. Whether these additions actually contribute to the recovery of active cell extracts has not been established. One milliliter of chilled cell slurry was added to a 2-ml screw-top plastic tube previously half-filled with beads and well chilled in a salt ice-water bath. The cells were homogenized twice for 1-min intervals at the high setting with intermittent cooling. The procedure was repeated several times to obtain three to four ml of pooled cell extract, to which a small amount of DNase (Sigma) was added.

Undisrupted cells and zirconium beads were removed at 4°C by anaerobic centrifugation at 11,500 × *g* for 10 min in an SS-34 rotor of an RC-5 Sorvall centrifuge (Sorvall, Norwalk, Conn.). The supernatant (crude extract) was centrifuged at 27,000 × *g* and 4°C for 60 min. The resulting membranous pellet (P27) was resuspended in anaerobic buffer containing 30% glycerol. The supernatant (S27) was either further centrifuged at 100,000 × *g* for 60 min at 4°C in a 70.1 Ti rotor of a Beckman L-2 ultracentrifuge or fractionated in the anaerobic chamber with Ultrafree-MC filter units (UFC3 LGC 00; Millipore Corp., Bedford, Mass.). These units employ an ultracellulose low-protein-binding membrane with a nominal molecular weight limit of 10,000. The resulting low-molecular-weight filtrate (the 10K filtrate) and the retentate of protein, concentrated about fourfold from the starting material, were stored in amber vials under argon at 4°C until use.

Protein concentrations were determined by the Coomassie blue method of Bradford (8) with bovine serum albumin as the standard. The proteins of cell suspensions were released by incubating appropriate dilutions in 0.1 N NaOH for 15 min in a boiling water bath before the assay.

Anaerobic O-demethylation reaction mixture and assay procedure. Unless otherwise stated, the reaction mixture was composed of a cocktail containing 2 mM hydroferulate, 1 to 2 mM THF, 2 mM ATP, 6.6 mM $MgCl_2$, 5 mM potassium phosphate (pH 7), and 2.7 mM dithioerythritol in a mixture of 12 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] and 60 mM Tris (both at pH 7.2). Note that the Tris was introduced with the THF, since it was used to elute THF from the QAE column. The source of enzyme was retentate, prepared as described above. For completeness, it should be pointed out that low levels of several other components were unavoidably present in the assay because they were present in the cell extract and hence also in the derived retentate. The effect of these additions on the activity assay has not been studied. They included low levels of glycerol (0.7%), pyruvate (0.135 mM), L-cysteine (0.0056%), and hydrocaffeate (2.3 μM). The final volume of the mixture was 100 to 200 μl. The level of retentate protein added was 0.7 to 1.0 mg/ml of reaction mixture.

Reactions were performed in the anaerobic chamber at 30°C under an atmosphere of N_2-H_2 (97:3). Cell extract was preincubated for 10 min in the buffer mixture already containing THF and half of the ATP-$MgCl_2$. The reaction was begun with the addition of a substrate mixture containing the hydroferulate and the balance of the ATP-$MgCl_2$. The reaction was terminated after 5 to 10 min by the addition of 1.0 ml of 0.1 N HCl, and the mixture was stored at −20°C aerobically until extracted for high-performance liquid chromatography (HPLC) analysis. Reactions were usually run in duplicate, and results are presented as the mean ± the standard deviation.

Quantitation of *O*-demethylated product. The acidified reaction mixture samples were applied to Bond Elute, C-8 octyl, solid-phase extraction columns (500-mg sorbent mass and 2.8-ml column volume; Analytichem International, Harbor City, Calif.) which had been conditioned as directed by the manufacturer by using a 20-place vacuum manifold (Applied Separations). The columns were then washed with 2 ml of 0.01 N HCl and eluted with 1 ml of methanol-acetonitrile (5:1). The eluate was diluted with an equal volume of 0.01 N HCl, and 40 μl of the resulting mixture was analyzed by HPLC with a model 332 Beckman instrument.

A Spherisorb C-18 reversed-phase column (25 cm by 4.6 mm; 5-μm particle size; Supelco, Bellefonte, Pa.) was run isocratically at 1 ml/min with a mobile phase composed of aqueous 5 mM formic acid (72.5%, vol/vol) and the methanol-acetonitrile mixture (27.5%, vol/vol). Eluting peaks were detected at 280 nm with a variable-wavelength UV detector (model 155; Beckman). Samples were injected automatically with a model 231-401 Auto-Sampling Injector (Gilson Medical Electronics, Middleton, Wis.). Peak heights were determined with an integrator (Chromjet; Spectra-Physics, San Jose, Calif.). The limit of detection by HPLC for hydrocaffeate or hydroferulate was 0.008 nmol. Thus, by using the sample preparation protocol described above, the limit of detection in an assay mixture was 4.0 μM. The approximate retention times for components of interest were 5.5 min for *p*-aminobenzoylglutamate, 7.3 min for hydrocaffeate, and 13.5 min for hydroferulate. The *p*-aminobenzoylglutamate is a degradation product of THF formed during the aerobic sample preparation procedure for HPLC.

RESULTS

Subcellular distribution of O-demethylating activity. Differential centrifugation of disrupted cell suspensions was used to obtain cytoplasmic (S27) and membrane (P27) fractions, and the cytoplasmic portion was further fractionated by microfiltration. The resulting retentate, which contained concentrated extract protein, and the 10K filtrate, which contained only low-molecular-weight components, were each tested for O-demethylating activity. Activity resided completely in the higher-molecular-weight protein fraction of the retentate rather than in the 10K filtrate.

The activity of intact cells and the S27 and P27 differential centrifugation fractions was initially evaluated on an equivalent-volume basis in order to compare the amount of activity present in cell extracts with that in intact cells and to examine the distribution of activity between cytoplasmic (S27) and membrane (P27) fractions. In order to assay the activity of the P27 fraction, the reaction mixture (see Table 1) was supplemented with 10K filtrate to provide any low-molecular-weight cofactors that were present in the S27 fraction but that would otherwise be deficient in the P27 fraction. Also, no attempt to wash the P27 pellet was made before the assay. The activity, expressed as nanomoles of product formed per minute per milliliter of cell suspension or extract was 309.5 ± 29.5 (mean ± standard deviation) for cells, compared with 105.0 ± 3.5 for the S27 fraction and 11.0 ± 0.5 for the P27 fraction, respectively. Thus, the cytoplasmic fraction was 34% as active as intact cells, whereas the membrane fraction was only 3.6% as active. When the activities of the subcellular fractions were normalized with respect to their protein content, the specific activity of the S27 cytoplasmic fraction was 14.2 ± 0.5 nmol/min/mg of protein, while that of the P27 membrane fraction was 3.8 ± 0.1 nmol/min/mg of protein. In additional experiments, a similar distribution of activity in subcellular fractions was observed with extracts prepared by French pressure cell disruption, and activity remained associated with the cytoplasmic supernatant upon further centrifugation at 100,000 × *g*.

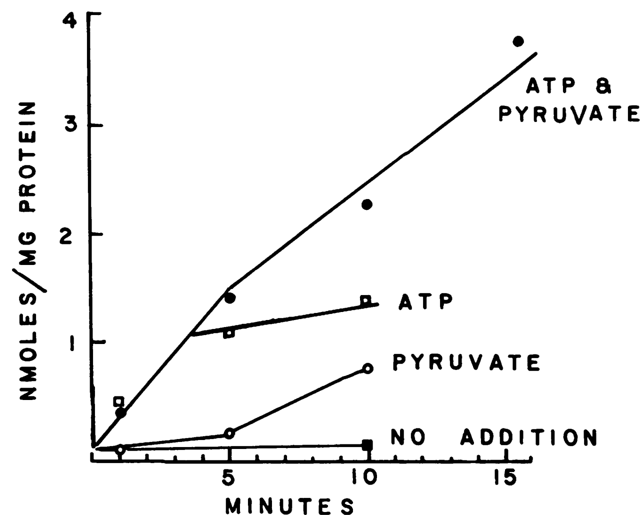

FIG. 1. Effects of including ATP or pyruvate in reaction mixtures lacking exogenous THF. The reaction mixtures were incubated at 30°C under an N_2-H_2 (97:3) atmosphere and contained 2 mM vanillate, 1.7 mg of S27 per ml, 6.25 mM $MgCl_2$, 0.3 mM $(NH_4)_2HPO_4$, and anaerobic buffer (100 mM Tris [pH 7.2], 2.3 μM $FeCl_2$, and 0.025% each cysteine and Na_2S). ATP (1 mM) and pyruvate (30 mM) were added as indicated. The product was protocatechuate, which was quantitated by HPLC.

Requirement for THF and ATP. Previous experiments (19) with vanillate (3-methoxy,4-hydroxybenzoate) as the substrate and no exogenous THF indicated that anaerobic O-demethylating activity in the S27 fraction, assayed under an N_2-H_2 (97:3) atmosphere in an anaerobic chamber, was dependent on ATP. The time course of the reaction showed that O-demethylation proceeded for only 1 to 5 min (Fig. 1). Furthermore, the presence of pyruvate in the reaction mixture allowed O-demethylation to proceed for an extended period but did not affect the initial rate of the ATP-dependent reaction. Pyruvate, in the absence of ATP, supported O-demethylation but only after a lag period. A carbon monoxide atmosphere (100%) could replace pyruvate in the activity assay. In this reaction mixture, which contained methoxylated substrate, ATP, pyruvate, and S27 but no exogenous THF, activity was somewhat variable, and the highest level observed was approximately 5% of that seen with intact cells. We hypothesized that the effect of pyruvate or carbon monoxide was indirect and might be related in part to the metabolism of an endogenous one-carbon carrier in extracts. The one-carbon carrier would most likely be THF or a corrinoid component.

This hypothesis was tested by supplementing reaction mixtures with THF or a reduced corrinoid, cob(II)alamin, in the presence or absence of pyruvate in order to determine the minimum requirements for the assay of activity. For this series of experiments, retentate was employed rather than the S27 fraction in order to decrease the level of endogenous metabolites introduced into the assay mixture with the addition of extract. It was also necessary for quantitation purposes to use a methoxylated substrate that is different from vanillate and would give rise to an O-demethylated

TABLE 1. Determination of minimum requirements for assay of anaerobic O-demethylating activity in *A. woodii* cell extracts

Assay mixture	Product formed in 10 min (nmol/mg)[a]
1. Complete RM[b]	108 ± 3
2. RM without THF	<4
3. RM without cob(II)alamin	99 ± 4
4. RM without cob(II)alamin and ATP	18 ± 3
5. RM without cob(II)alamin and pyruvate	103 ± 30
6. RM without cob(II)alamin, pyruvate, and ATP	<4

[a] Reaction mixtures were incubated for 10 min at 30°C in an anaerobic chamber under an atmosphere of N_2-H_2 (97:3). Each values is the mean ± standard deviation of duplicate reactions.

[b] Complete reaction mixture (RM) contained 20 μM cob(II)alamin, 0.8 mM THF, 2 mM ATP, and 30 mM pyruvate; the enzyme source was retentate (1 mg/ml). No enhancement of activity was observed if complete RM was further amended with 0.4 mM CoA alone or in combination with a 0.5 mM concentration of either NAD^+, $NADP^+$, NADH, NADPH, flavin mononucleotide, or flavin adenine dinucleotide.

product that could be well separated from *p*-aminobenzoylglutamate by HPLC. Hydroferulate satisfied this criterion. Assays were performed in the anaerobic chamber under an atmosphere of N_2-H_2 (97:3). From a comparison of the data presented in lines 1 and 2 in Table 1, it can be seen that activity is dependent on the presence of THF and that omitting cob(II)alamin seems to have no effect on the THF-dependent activity (line 3). The dependence on ATP is evidenced in lines 4 and 6. In the presence of both ATP and THF, pyruvate seems unnecessary (line 5). CO was not tested for possible effects on the ATP- and THF-dependent O-demethylating activity. No further stimulation of activity was observed with the addition of CoA, pyridine nucleotides, flavin mononucleotide, or flavin adenine dinucleotide. Thus, O-demethylation was subsequently assayed with reaction mixtures containing hydroferulate, ATP, and THF as described in Materials and Methods.

Characteristics of the anaerobic O-demethylating activity assayed in the standard reaction mixture. The time course for product formation in a representative experiment with retentate as the enzyme source is shown in Fig. 2A. The rate of reaction was linear for at least the first 5 min and became lower thereafter. Thus, in subsequent work, reactions were allowed to proceed for only 5 min. The relationship between velocity and the amount of retentate added to the reaction mixture is shown in Fig. 2B. Serial twofold dilutions of retentate were made by using the 10K filtrate as a diluent in order to provide a constant level of endogenous low-molecular-weight cofactors. The velocity of the reaction varied with the amount of protein added; however, a sensitivity to dilution was seen at low levels of retentate, i.e., when the protein concentration was <1 mg/ml, for which the O-demethylating activity was lower then expected for a linear relationship.

Determination of apparent K_m values. Reaction velocity was examined as a function of the concentration of the methoxylated substrate as well as those of ATP and THF. The concentration of each was varied in the presence of saturating levels of the other two. Reactions were run in duplicate, and the mean velocity ± the standard deviation was determined. An Eadie-Hofstee plot (v versus v/S, where v is velocity and S is substrate concentration) of the data was used to estimate the V_{max} and K_m parameters for each variable. These calculated parameters were used to draw the

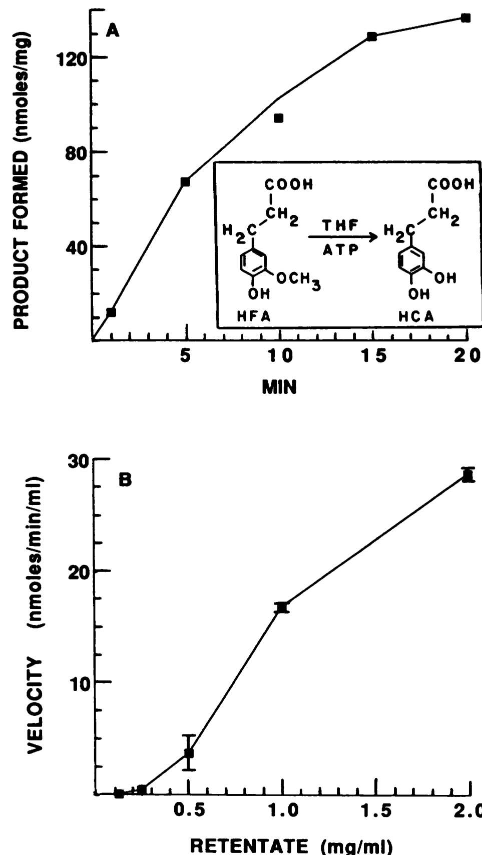

FIG. 2. Initial characterization of the anaerobic O-demethylation assay with retentate as the enzyme source. (A) Progress curve for product formation versus time of incubation. The chemical structures for the aromatic substrate and product in the anaerobic O-demethylation assay, hydroferulic (HFA) and hydrocaffeic (HCA) acids, respectively, are shown in the inset. (B) Dependence of velocity on amount of retentate. Assay conditions were as described in the legend to Fig. 3.

curves in Fig. 3. Also plotted are the observed data for mean velocity.

For the methoxylated substrate, a V_{max} of 15.4 nmol/min/mg of protein and an apparent K_m of 0.65 mM hydroferulate were estimated. The saturation curve and Lineweaver-Burk reciprocal plot are shown in Fig. 3A. In the experiments with variable ATP concentrations, the level of $MgCl_2$ was maintained at 6.6 mM, except at the highest ATP concentration, where it was increased to 13.2 mM. Also, reactions were initiated with the combined addition of hydroferulate and ATP, and ATP was omitted from the preincubation step. A V_{max} of 15.3 nmol/min/mg with an apparent K_m of 0.27 mM ATP was estimated (Fig. 3B). For variable THF, the V_{max} was estimated as 17.1 nmol/min/mg, with an apparent K_m of 0.17 mM THF (Fig. 3C).

Physical characteristics of the enzyme system. The O-demethylating activity appeared to be stable when the retentate was stored in 15% glycerol at 4°C in an amber vial under an argon atmosphere, since there was little loss of activity after 6 to 8 days with either the S27 or the retentate under these conditions. The O-demethylating activity was inactivated by heat treatment, as observed by incubating retentate in the anaerobic chamber at 60°C for 30 min before assaying it for activity. Only 30% of the starting activity remained. By

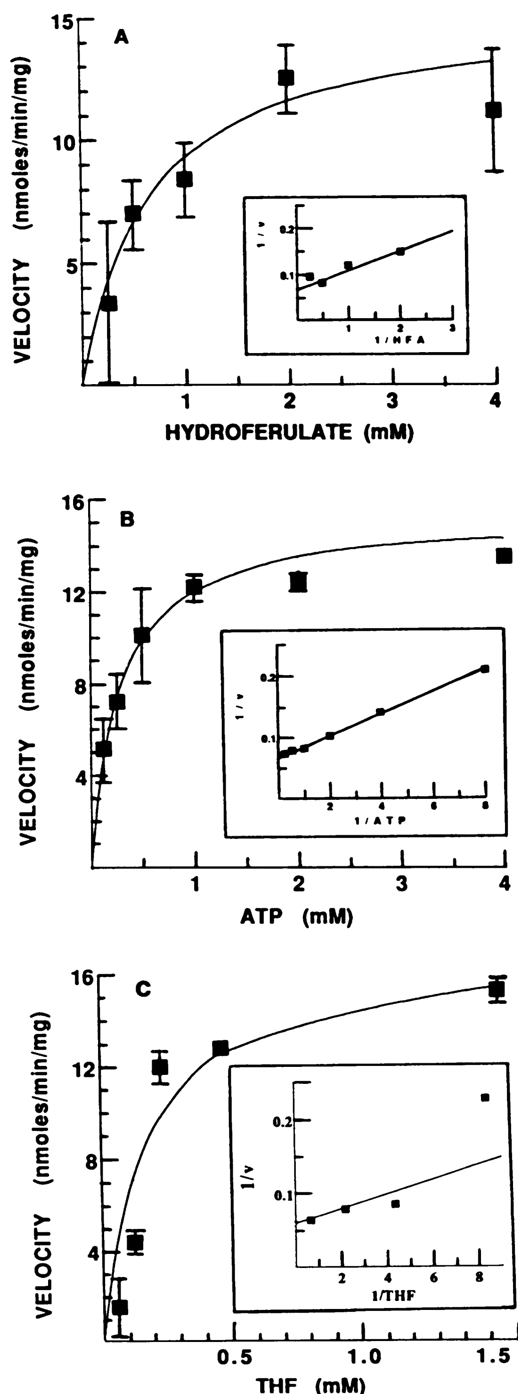

FIG. 3. Observed velocity (v) versus substrate concentration curves for hydroferulate (HFA), ATP, and THF. Insets show reciprocal plots of the data. Unless otherwise noted, standard reaction mixtures included 2 mM hydroferulate, 0.92 mM THF, and 2 mM ATP with retentate present at 1 mg/ml. The mixtures were incubated for 5 min at 30°C at pH 7.2 in a Coy anaerobic chamber with an N_2-H_2 (97:3) atmosphere. Datum points are means, and error bars indicate standard deviations.

contrast, retentate incubated under the same conditions but at room temperature showed no loss of activity. O-demethylating activity was abolished by flushing gently stirred retentate with water-saturated air for 30 min at room temperature before the assay.

DISCUSSION

On the basis of the present work, we have established that the O-demethylating system of *A. woodii* is dependent on THF and ATP (Table 1 and Fig. 3). The O-demethylating activity does not appear to be membrane associated. The observation that activity is sensitive to dilution (Fig. 2B) could suggest that a dissociable enzyme system catalyzes the anaerobic O-demethylation reaction. Since the THF- and ATP-dependent assay is unaffected by the presence of pyruvate and does not require CO, the reaction is likely to be limited to the O-demethylation event itself rather than being obligately linked to a chain of other enzymic reactions in extracts, such as acetyl-CoA synthase–CO dehydrogenase activity (12, 16, 36). The assay, with HPLC analysis of product formation, is applicable to other methoxylated substrates, as long as the demethoxylated product can be analytically detected and quantitated. It is hoped that the assay developed with *A. woodii* extracts will be useful in O-demethylation studies with other acetogens. The apparent K_m of 0.65 mM observed for hydroferulate is in the same range as the K_m previously determined for vanillate (0.4 mM) in the S27 fraction of *A. woodii* cell extracts assayed under an N_2-H_2 (97:3) atmosphere in the presence of added ATP, but with pyruvate replacing exogenously added THF (19). It is also similar to the 0.5 mM K_m for syringate (4-hydroxy,3,5-dimethoxybenzoate) measured for intact cells of *C. thermoaceticum* (36).

Ethers are relatively stable organic compounds (30). Aerobic bacteria use hydroxylating enzymes to cleave *O*-methyl ether linkages (7, 9, 33), and O-demethylation also results from the action of fungal lignin peroxidases and laccases (25). The bacterial hydroxylating enzymes require NADH and O_2, and the products of the reaction are NAD^+, formaldehyde, and the hydroxylated aromatic derivative. By contrast, the anaerobic O-demethylating reaction is dependent on THF and ATP but independent of pyridine nucleotide cofactors and must therefore be mechanistically distinct from the action of the aerobic hydroxylating enzymes.

It is likely that THF and ATP not only are necessary for O-demethylating activity but in fact are cosubstrates. This can be established only by identifying the products derived from ATP and THF and determining the stoichiometry of reactants and products. Before these studies can proceed, it may be necessary to work with an at least partially purified enzyme preparation. It is interesting that Doré and Bryant (16) found no stimulation of anaerobic O-demethylating activity upon the addition of THF to cell-free preparations from *Syntrophococcus sucromutans*. The fact that ATP was not added to their reaction mixtures, and therefore may have been limiting, might account for the failure of THF to stimulate O-demethylation in this system. Identifying the one-carbon THF product is particularly important. Possible candidates are methyl-THF and methylene-THF. In reactions involving the activation of methyl groups in the dissimilation of serine or glycine, methylene-THF is the product formed (29). The methylene-THF–methyl-THF coupling, in which the two compounds are interconverted in acetogens by a membrane-associated methylene-THF reductase, is a reaction exergonic enough to be involved in energy conser-

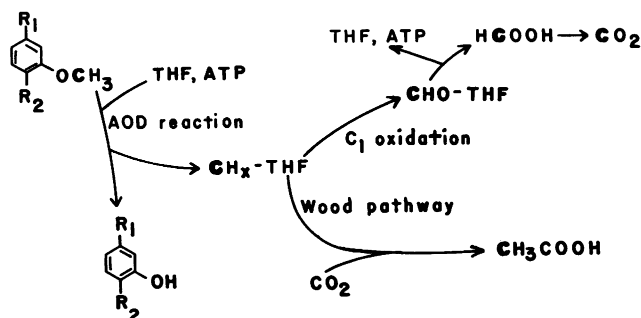

FIG. 4. Carbon flow scheme for acetogens utilizing *O*-methyl substituents. Carbon derived from the *O*-methyl group is shown in boldface.

vation through a chemiosmotic mechanism (15, 23). If methylene-THF is the product of the O-demethylation reaction, an additional reactant serving as an electron acceptor may be involved.

Aspects of the metabolism of *O*-methyl substituents by acetogens are summarized in Fig. 4. Evidence from several sources suggests that anaerobic O-demethylating enzymes may have broad specificity with respect to aromatic acids (14) as well as other aromatic compounds (12), including guaiacol (Fig. 4, R_1=H, R_2=OH) and chloroguaiacols (20). Work with purified enzymes will be necessary to determine whether certain strains of acetogens encode multiple O-demethylating enzymes, as has been suggested in some studies with *E. limosum* ATCC 8486 (14) and *A. woodii* ATCC 29683 (6). Acetogens growing on methoxylated compounds are expected to convert some of the methyl substituents to the methyl carbon of acetate while oxidizing others to carbon dioxide. This has in fact been observed in growth studies with strain TH-001, an acetogen taxonomically related to *A. woodii* and *E. limosum* (37). When *O*[*methyl*-^{14}C]vanillate was used as the growth substrate, labeled $^{14}CO_2$ as well as [^{14}C]acetate was produced (18); labeling occurred predominantly in the methyl carbon (unpublished results). Thus, the dissimilation of *O*-methyl substituents to CO_2 and acetate in many acetogens appears similar to the metabolism of ^{13}C-labeled methanol to CO_2 and methyl-labeled acetate (24). Acetate is derived from acetyl-CoA; it is synthesized by the acetyl-CoA–CO dehydrogenase reaction that is central to the Wood pathway (35). In studies of the utilization of *O*-methyl substituents, CO_2 is often used as the electron acceptor and is the precursor to the carboxyl carbon of acetate. In the presence of alternate electron acceptors, less acetate is formed and thus proportionately more carbon should be metabolized through the one-carbon oxidation route. The alternate electron acceptors can include the propenoate side chain of ferulate or cinnamate in *A. woodii* (3, 21, 34) and thiosulfate or dimethyl sulfoxide in *C. thermoaceticum* (5).

ATP is necessary for the O-demethylation reaction, and it can be regenerated by substrate-level phosphorylation in the formyl-THF synthetase reaction (i.e., in the one-carbon oxidation route of Fig. 4) or in the acetate kinase reaction (i.e., in the terminal step of acetate production from the acetyl-CoA synthesized by the Wood pathway). However, a mechanism for net ATP synthesis is necessary for growth on methoxylated substrates. If methylene-THF is formed in the anaerobic O-demethylation reaction, additional energy might be conserved because of the methylene-THF reductase reaction.

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