Anaerobic Pathway for Conversion of the Methyl Group of Aromatic Methyl Ethers to Acetic Acid by *Clostridium thermoaceticum*[†,‡]

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ABSTRACT: *Clostridium thermoaceticum* and other anaerobic acetogenic bacteria can utilize the methyl group of aromatic methyl ethers as a carbon and energy source. It has been unclear what pathway is used to metabolize this methyl group. In the work reported here, the pathway was established by identifying and quantitating the substrates, stable intermediates, and products of O-demethylation of syringic acid. By measuring the dependence of the O-demethylation reaction on purified enzymes of the acetyl-CoA pathway, it was established that CO dehydrogenase, the corrinoid/iron–sulfur protein, and methyltransferase all were required for acetyl-CoA formation. By ^{13}C-NMR spectroscopy it was shown that the O-demethylase from *C. thermoaceticum* converts the methyl group of syringate to methyltetrahydrofolate (CH_3-H_4folate). When the reaction was conducted in the presence of CO, H_2, or titanium(III), or in the absence of any electron donor, the rate of demethylation of syringic acid at pH 7.2 was ~15 nmol min^{-1} mg^{-1}. In the absence of CO, CH_3-H_4folate accumulated as a stable product. When CO was added, $^{13}CH_3$-H_4folate was converted to [2-^{13}C]acetyl-CoA, [2-^{13}C]acetyl phosphate, and [2-^{13}C]acetate. Therefore, the acetogenic O-demethylase uses H_4folate as acceptor of the methyl group of phenyl methyl ethers and catalyzes the formation of CH_3-H_4folate. The pathway of conversion of CH_3-H_4folate, CO, and CoA to acetyl-CoA has been studied previously. Methyltransferase catalyzes the reaction of CH_3-H_4folate with the corrinoid/iron–sulfur protein to form a methylcobalt species. The nickel/iron–sulfur enzyme CO dehydrogenase then catalyzes the final steps in the formation of acetyl-CoA. Although *C. thermoaceticum* is a strict anaerobe, the O-demethylase activity in cell extracts was found to be relatively oxygen insensitive and to not require reductive activation. It also was insensitive to propyl iodide, indicating that it is not a corrinoid protein. Having a broad substrate range, the O-demethylase can demethylate lignin-derived methoxy phenolics such as syringic or hydroxyvanillic acids, plant hormones like acetosyringone, and herbicides such as dicamba to the corresponding phenolic products.

The global carbon cycle involves the oxidation of approximately 200 billion tons of biomass carbon to CO_2 every year. Much of the earth's biomass is made up of polymers such as lignin and cellulose. Lignin is a complex aromatic polymer that comprises 17–33% of wood and constitutes ~ 25% of the earth's renewable biomass. The biodegradation of lignin requires its conversion to monomeric units, mostly by fungi. Phenyl methyl ethers such as syringic acid are the major product of lignin depolymerization. Industry also generates a number of aromatic methyl ethers for use as pharmaceuticals, reagents for synthesis, herbicides, and solvents. These compounds can be metabolized to reduce the concentrations below environmentally harmful levels. For example, dicamba, 3,6-dichloro-2-methoxybenzoate, is a widely used broadleaf herbicide which can be degraded by aerobic (Krueger *et al.*, 1989) and anaerobic (Taraban *et al.*, 1993) bacteria. Removal of the *O*-methyl moiety is an important step in the biodegradation of phenyl methyl ethers.

A number of anaerobic bacteria can metabolize the methyl group of methoxylated aromatic compounds. The first anaerobes shown to cleave ether linkages were in an enrichment culture containing methanogens (Healy & Young, 1979). The acetogen *Acetobacterium woodii* was selectively isolated from freshwater mud and sewage sludge samples with the methoxylated aromatics anisol and trimethoxybenzoate as substrates (Bache & Pfennig, 1981). These compounds were used as carbon and electron sources, and acetic acid was the sole fermentation product (Bache & Pfennig, 1981). Even better growth yields were found on the methoxylated aromatic than on methanol (Tschech & Pfennig, 1984). Other anaerobes, including *Clostridium thermoaceticum* (Daniel *et al.*, 1988), have been found that can grow using the methoxyl group as an energy source (Genther *et al.*, 1981; Kreft & Schink, 1993; Krumholz & Bryant, 1986; Messmer *et al.*, 1993; Schink *et al.*, 1992; Stupperich & Konle, 1993).

Mechanistic studies on anaerobic O-demethylation of phenyl methyl ethers are limited. We chose to study the pathway of O-demethylation in *C. thermoaceticum*, the organism in which the enzymes of the reductive acetyl-CoA pathway have been most thoroughly studied. After *C. thermoaceticum* was shown to grow on methoxylated aromatics (Daniel *et al.*, 1988), the enzyme system was shown to be inducible by methoxylated aromatics and repressed by glucose and methanol (Wu *et al.*, 1988). Besides syringic acid, several other methoxylated aromatics were shown to be demethylated by *C. thermoaceticum* including vanillate; 5-hydroxy- and 2-methoxyvanillate; 1,2,3-trimethoxy- and 1,2-, 1,3-, and 1,4-dimethoxybenzene; and 2-methoxyphenol (Wu *et al.*, 1988). Important questions that remain unanswered include, what is the initial methyl acceptor in the pathway? and at what step in acetyl-CoA synthesis does the methyl group enter? The oxygen atom was shown to be retained in the phenolic product (DeWeerd *et al.*,

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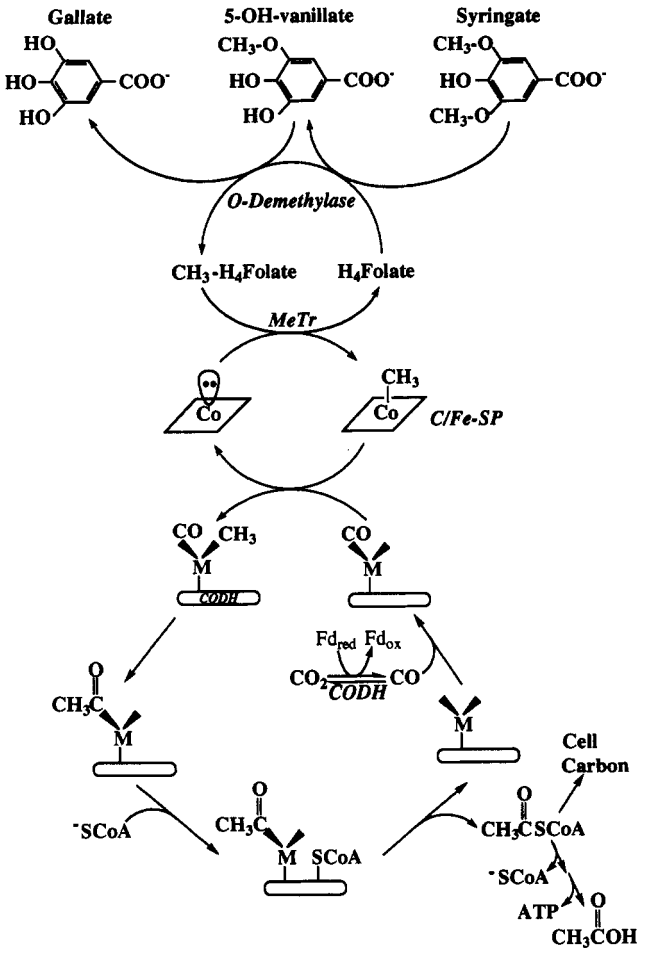

FIGURE 1: Pathway of conversion of the methyl group of syringic acid to the methyl group of acetyl-CoA.

Table 1: Products of Syringic Acid Metabolism Identified by Thin-Layer Chromatography

compound	R_f[a]	color reaction with $FeCl_3$
syringic acid	0.23	brick red
gallic acid	0.03	dark blue
5-hydroxyvanillate	0.12	blue
pyrogallol	0.06	grayish green

[a] Mobility relative to the solvent front.

1988), indicating that this reaction is catalyzed by an O-demethylase, not a demethoxylase. O-Demethylation in *A. woodii* was shown to require tetrahydrofolate (H_4folate) (Berman & Frazer, 1992), indicating that methyltetrahydrofolate (CH_3-H_4folate) was the likely product. In these studies, metabolism of the aromatic methyl ether was followed; the methylated product was not identified. In a seemingly contradictory study, H_4folate inhibited acetate formation from vanillate by *Syntrophococcus sucromutans* (Doré & Bryant, 1990). Therefore, it became unclear whether H_4folate plays a role in the formation of acetyl-CoA or diverts methyl flux from acetyl-CoA formation (Kreft & Schink, 1993). The different routes for entry of the methyl group into the acetyl-CoA pathway can be summarized. It is possible that the methyl group enters (i) by reaction with H_4folate to form CH_3-H_4folate, (ii) by direct reaction with the corrinoid/iron–sulfur protein (C/Fe-SP) or carbon monoxide dehydrogenase (CODH) to form methyl–protein adducts thus bypassing the methyltransferase (MeTr) step, or (iii) by reaction with a so-far-undetected methyl acceptor. In the work reported in this paper, an approach using ^{13}C-NMR spectroscopy was developed to identify and quantitate the substrates, stable intermediates, and products of O-demethylation. We show that, in *C. thermoaceticum*, H_4folate plays a role as the initial coenzymic acceptor of the methyl group of the phenyl methyl ether. Our results (Figure 1) demonstrate that the O-demethylation reaction is catalyzed by an oxygen-stable enzyme that converts the methyl group of syringate and other phenyl methyl ethers to CH_3-H_4folate and gallic acid. CH_3-H_4folate, CO, and CoA are then converted to acetyl-CoA by the enzymes of the acetyl-CoA pathway.

MATERIALS AND METHODS

Cultivation of Clostridium thermoaceticum and Cell Extract Preparation. C. thermoaceticum was grown at 55 °C with 100% CO_2 as gas phase in a medium similar to that described earlier (Wu *et al.*, 1988). Cells were grown in 60 L of medium containing 20 mM syringate and 10 mM glucose. The medium contained (in grams per liter) yeast extract, 5.2; tryptone, 5.2; ammonium sulfate, 1; magnesium sulfate heptahydrate, 0.26; ferrous diammonium sulfate hexahydrate, 0.04; cobalt nitrate hexahydrate, 0.032; disodium tungstate dihydrate, 0.026; disodium molybdate dihydrate, 0.0192; disodium selenite, 0.0016; nickel chloride, 0.0168; sodium thioglycolate, 0.52; nitrilotriacetic acid, 0.078; sodium bicarbonate, 18.4; dipotassium phosphate, 7.7; and monobasic potassium phosphate, 6. A vitamin mixture was included (Wu *et al.*, 1988). The cell yield was 2 g/L (wet weight).

Growth was monitored by measuring the optical density at 600 nm. The syringic acid and gallic acid concentrations were routinely estimated by performing thin-layer chromatography on silica gel plates. Samples were taken at different time points during growth, quenched by adding 1 N HCl, and centrifuged at 10000*g* for 3 min. The clear supernatant was extracted three times with equal volumes of diethyl ether, and the ether extracts were pooled and evaporated to dryness. The residue was dissolved in a minimum volume of ethanol and analyzed by TLC on silica gel 60 plates using the organic phase of a toluene:acetic acid:water (6:7:3, v/v) mixture as mobile phase. The separated products were visualized by use of a UV lamp and identified by their relative mobility values (Table 1). The TLC plates were also sprayed with 1% $FeCl_3$ to identify the substrates and products by their characteristic color reactions (Table 1).

Anaerobic cell extracts were prepared by suspending 20 g of cells in 40 mL of solution containing 0.1 M sodium/potassium phosphate, pH 7.2; 0.5 mM syringic acid; 8 mM $MgCl_2$; 3 mM pyruvate; 1 mM dithionite; and 1 mM dithiothreitol (DTT). They were disrupted inside a Coy Laboratory Products anaerobic chamber (Ann Arbor, MI) with a Heat Systems sonicator and centrifuged for 60 min at 30 000 rpm in stainless steel centrifuge tubes with a type 35 rotor (Beckman Instruments, Inc.). The supernatant (cell extract) was stored anaerobically at −80 °C until use. Protein concentration was determined by the Rose Bengal dye-binding assay (Elliott & Brewer, 1978) using lysozyme as standard and was found to be 43 mg/mL.

Synthesis of $^{13}CH_3$-Labeled Syringate. The two-step synthesis of [^{13}C]syringic acid was performed essentially as described (Roth *et al.*, 1982). First, $^{13}CH_3$-labeled trimethoxybenzoic acid was synthesized from *n*-propyl gallate and [^{13}C]iodomethane (1.12 g of *n*-propyl gallate, 1.15 g of KOH, and 4 g of $^{13}CH_3I$ in 7.5 mL of dimethylformamide). Next, [^{13}C]trimethoxybenzoic acid was converted to [^{13}C]-syringic acid in concentrated sulfuric acid at 40 °C. Product analysis by ^{13}C-NMR showed a major resonance at 56.37

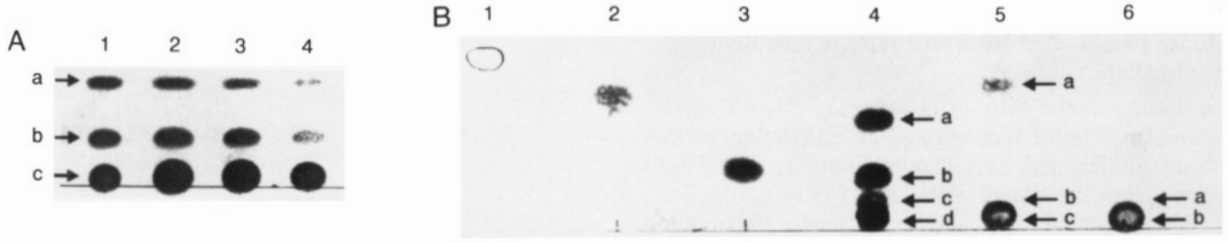

FIGURE 2: (a) Thin-layer chromatography of the O-demethylation reaction. Cell extracts were treated anaerobically (lanes 1 and 2) or exposed to oxygen for 30 min (lanes 3 and 4). Reactions were performed in the presence of CO (lanes 2 and 3) or in the absence of any reductant (lanes 1 and 4). The arrows designate (a) syringate, (b) hydroxyvanillate, and (c) gallate. (b) Thin-layer chromatography of mixtures containing CO, 10 mM H_4folate, 6 mM ATP, 6 mM $MgCl_2$, 1 mg of cell extract, and either syringate (lane 4), dicamba (lane 5), or acetosyringone (lane 6) after 6 h of reaction. Lanes 1–3 contain the standards dicamba, 3,6-dichlorosalicylate, and acetosyringone, respectively. Lane 4: (a) syringate, (b) hydroxyvanillate, (c) pyrogallol, and (d) gallate. Lane 5: (a) 3,6-dichlorosalicylate and (b, c) further metabolites. Lane 6: (a, b) O-demethylation products of acetosyringone.

ppm due to [^{13}C]syringic acid and a minor peak at 56.16 ppm due to 3,4,5-trimethoxybenzoate. Since the intensity of the 56.16 ppm peak was 5% relative to that of the syringate peak, the [^{13}C]syringic acid was $\sim$ 95% pure.

Assay of the O-Demethylase. The O-demethylation reaction was performed in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.2), 2 mM $MgCl_2$, 2–3.5 mM H_4folate [(6*RS*)-5,6,7,8-tetrahydrofolic acid trihydrochloride], 2 mM CoA, 2 mM ATP, 2–13 mM syringate, 0.15 mg of CODH, 0.27 mg of C/Fe-SP, and 0.1 mg of MeTr in a total volume of 150–200 μL. The reaction was initiated with 30 μL of cell extract. Reactions were performed in the anaerobic chamber at 45 °C and terminated by quenching aliquots in an equal volume of 2 N HCl. The quenched mixture was then frozen instantly in liquid nitrogen and stored at –80 °C. In some cases, the reaction mixture was bubbled with CO or H_2 (1 atm) before addition of cell extract. Some reactions were performed in an electrochemical cell described earlier (Lu & Ragsdale, 1991) using a potentiostat (Model CV-1A, Bioanalytical Systems Inc.) to apply the potential. A voltmeter (Beckman, Model 4410) was connected across the working and reference electrodes to monitor the potential. Aliquots (20 μL) of the reaction mixture were removed from the electrochemical cell under a high positive nitrogen gas flow. The reaction was also studied by a continuous recording spectrophotometric assay that included all the components described above plus 2 mM titanium citrate. CODH (Ragsdale & Wood, 1985), C/Fe-SP (Ragsdale *et al.*, 1987), and MeTr (Drake *et al.*, 1981) were purified as described earlier. H_4folate was purchased from Dr. B. Schircks Laboratories, Switzerland.

NMR Experiments. The reaction mixture was as described above except that $^{13}CH_3$-labeled syringate was used. The reaction was stopped at different time points by quenching 150 μL of reaction mixture into 50 μL of 1 N HCl. The sample was then centrifuged, and the supernatant was added to a methanol solution in D_2O. The chemical shifts were calculated using methanol (49.00 ppm relative to TMS) as an internal standard and are reported relative to TMS. The chemical shifts of acetyl-CoA, CH_3-H_4folate, and acetic acid were assigned with standards and acetyl phosphate by calculations based on the expected shift relative to acetate. Relative concentrations were determined by comparing the peak intensities to those of the methanol internal standard. Absolute concentrations were obtained by comparing the intensity of an individual standard of known concentration to that of methanol run under identical conditions. The ^{13}C-NMR data were acquired on a General Electric Omega-500 NMR spectrometer, operating at 125.749 MHz. Data were acquired using a 45° observe pulse (7.5-μs pulse width) with broad-band proton decoupling via the GARP1-6 modulation scheme. The relaxation delay for signal averaging purposes was 2.0 s per scan.

RESULTS

Enzymes involved in the metabolism of phenyl methyl ethers are derepressed by their aromatic substrates. We found that addition of glucose at concentrations $\leq$ 10 mM greatly increased the cell yield and did not repress the synthesis of these enzymes. When cells were harvested during exponential phase at an optical density at 600 nm of 2.05, the cell yield was $\sim$2 g per liter of medium. This is approximately 10-fold higher than could be attained with growth in the absence of glucose. Cells grew to a final OD of 3.5 before the syringate was fully utilized.

Study of the O-Demethylase by Thin-Layer Chromatography (TLC). In order to determine the oxygen sensitivity of the O-demethylase, the reaction was run in the presence of CoA; H_4folate; purified CODH, C/Fe-SP, and MeTr; and either anaerobically prepared cell extract (Figure 2a, lane 1) or cell extract that had been exposed to air for 30 min (lane 4). Incubation of the cell extract with oxygen did not decrease the rate of demethylation of syringate [a] to form the monomethoxylated intermediate 5-hydroxyvanillate [b] and the dihydroxylated product gallic acid [c], indicating that the O-demethylase is not oxygen sensitive.

The O-demethylase from TMBS 4 (Kreft & Schink, 1993) was reported to require reductive activation. When the reaction described above was performed in the presence of Ti(III) citrate, under H_2, or in an electrochemical cell in the presence of methyl viologen, the rate of O-demethylation was similar to the rate observed when the reaction was performed under an atmosphere of CO (lanes 2 and 3). Furthermore, there was only a slight decrease in the rate when the reaction was performed in the absence of reductant with cell extract that had been exposed to oxygen (lane 4). These experiments demonstrate that reductive activation of the O-demethylase is not required.

The O-demethylase was found to utilize a broad range of substrates (Figure 2b). As shown in lane 4, syringate [a] was converted to hydroxyvanillate [b] and then to gallate [d]. Gallate was further decarboxylated to give pyrogallol [c]. Dicamba (3,6-dichloro-2-methoxybenzoic acid) (lane 5) and acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) (lane 6) were also used as substrates. Dicamba (lane 5) was demethoxylated to yield 3,6-dichlorosalicylate [a] and then was further metabolized. Although we have not further analyzed the final product [c], based on the basis of a similar mobility to gallic acid in this solvent system it is possible that

dechlorination has occurred. Demethylation of acetosyringone (lane 6) led to a product [b] with a relative mobility similar to that of gallate.

Quantitation of the Rate of O-Demethylation by Spectrophotometry. The products of the O-demethylation reaction (5-hydroxyvanillate, gallate, and pyrogallol) are vicinal diols that form yellow complexes with Ti(III) (Kreft & Schink, 1993). The extinction coefficients at 450 nm for the titanium complexes of 5-hydroxyvanillate, gallate, and pyrogallol are 2.5, 2.6, and 1.8 mM^{-1} cm^{-1}, respectively (Kreft & Schink, 1993). On the basis of the fact that syringate does not complex Ti(III), a continuous recording assay of the O-demethylase activity was developed in which the absorption at 450 nm is followed as a function of time. The reaction rate was linear with concentration of cell extract, and the specific activity of the O-demethylase was determined to be 15 nmol min^{-1} mg^{-1}. This quantitative assay was consistent with the rate of O-demethylation estimated by TLC.

Identification and Quantitation of O-Demethylation Products by ^{13}C-NMR. To determine where in the acetyl-CoA pathway the methyl group enters, the O-demethylation of [^{13}C]syringic acid was followed by ^{13}C-NMR in the presence and absence of CO (Figures 4 and 5). When the reaction was performed in the absence of CO in an NMR tube (Figure 3a), $^{13}CH_3$-H_4folate (peak c) accumulated. The rate constants for demethylation of syringate and formation of CH_3-H_4-folate (Figure 3b) were equivalent, 0.02 ± 0.005 min^{-1}. Addition of CoA and the purified enzymes CODH, C/Fe-SP, and MeTr had no effect on this reaction.

Identification of the product of the reaction as $^{13}CH_3$-H_4-folate was unambiguous. At pH 7, both the product and the standard CH_3-H_4folate have chemical shifts at 42.7 ppm (Figure 3a). The pK_a value for the N-5 of CH_3-H_4folate is 5.0 (S. Zhao and S. W. Ragsdale, unpublished). When the reaction was quenched by acid and NMR was performed on the acidified reaction mixture (pH ~ 1.0), the N-5 was fully protonated and the chemical shift of the methyl group was observed at 44.9 ppm. At pH 1.0 and at 25 °C as in Figure 4, the line width was 50 Hz. As the temperature was increased, the line width decreased to a limiting value of 6 Hz (at $T = \infty$) (Figure 3c). The temperature dependence of the line width is an effect on T_2 relaxation since the line width was independent of the delay time when it was varied from 0.3 to 5 s. The rather long T_2 at 25 °C is likely to result from a chemical exchange broadening mechanism in which the methyl group moves in an umbrella-like fashion between apical and equatorial positions at rates that depend upon temperature and pH. At pH 7.0, the line width was 8 Hz. At this pH, the N-5 is deprotonated, causing the methyl group to undergo faster chemical exchange resulting in a narrow line width. Identical behavior was observed with authenic CH_3-H_4folate.

In another experiment, the reaction was performed in the presence of 2.0 mM syringate in the absence of CO. At various times, the mixture was quenched with acid, methanol was added as an internal standard, and the spectrum was recorded. Since the volume was increased by 3.3-fold, the actual concentrations of ^{13}C-labeled compounds in the reaction mixture were 3.3-fold greater than those plotted versus time in Figure 4b. After 180 min, the concentration of CH_3-H_4-folate (Figure 4a, peak c; Figure 4b, △) reached 2.1 mM (0.63 mM in the NMR tube), accounting for 52% of the methyl groups present initially as syringate (peak a; ●). The rate of CH_3-H_4folate formation was the same as the rate of syringate depletion. Hydroxyvanillate could clearly be distinguished from syringate on an expanded scale and was observed as a

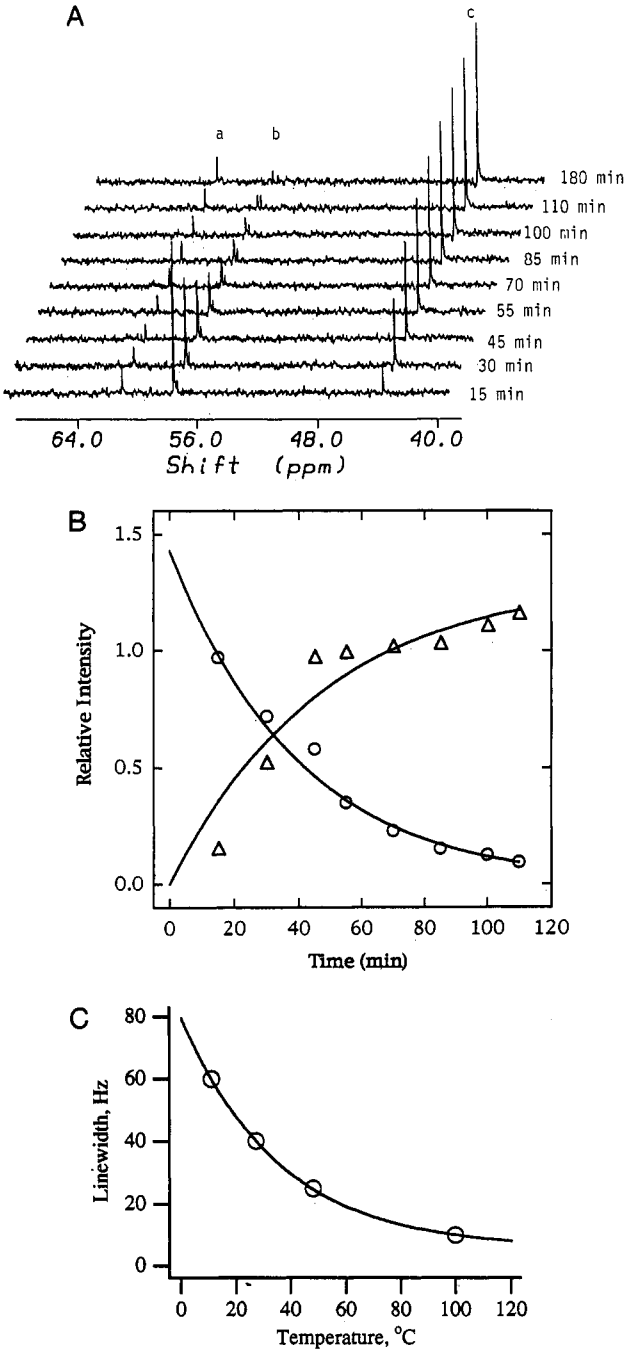

FIGURE 3: (a) ^{13}C-NMR study of the O-demethylation of syringate. The reaction mixture contained syringate (3.5 mM), H_4folate (6.5 mM), ATP (3.5 mM), $MgCl_2$ (3.5 mM), and cell extract (4 mg). The reaction was performed in the NMR tube at 45 °C. Peaks: a, a buffer peak used as an internal standard; b, syringate; c, CH_3-H_4-folate. (b) Relative intensities of syringate (○) and CH_3-H_4folate (△) as a function of time. The data were fit to single exponentials with rate constants of –0.024 ± 0.004 min^{-1} and 0.021 ± 0.005 min^{-1} for syringate depletion and CH_3-H_4folate formation, respectively. (c) Temperature dependence of the line width of the $^{13}CH_3$-H_4folate peak at pH 1.0.

transient intermediate (○). After CO was introduced at 180 min, CH_3-H_4folate was converted to [2-^{13}C]acetyl-CoA (peak d; □), acetyl-phosphate (peak f, ▽), and acetic acid (peak e, ◇). After 20 h, the syringate was completely consumed and the sum of the concentrations of acetyl-CoA, acetyl phosphate, and acetate amounted to 1.3 mM acetyl groups. Since this is equivalent to 4.2 mM acetyl groups in the reaction mixture, the 4.0 mM methyl groups initially present in 2.0 mM syringate can be accounted for in the acetylated products of the reaction.

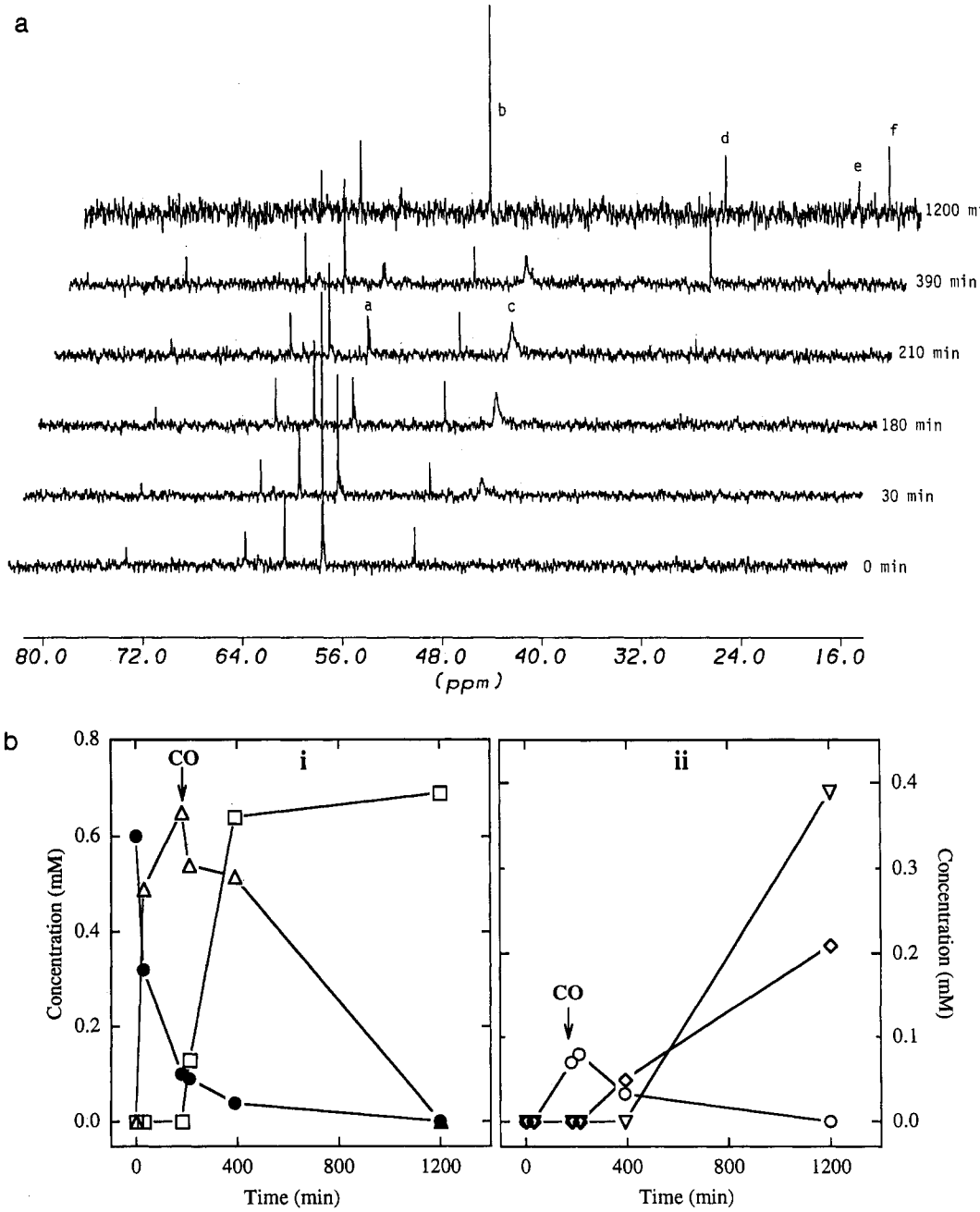

FIGURE 4: (a) ^{13}C-NMR study of the O-demethylation of syringate. The reaction mixture contained syringate (2 mM), H_4folate (3.5 mM), ATP (2 mM), $MgCl_2$ (2 mM), CoA (2 mM), CODH (0.4 mg), MeTr (0.4 mg), C/Fe-SP (1 mg), potassium phosphate buffer (100 mM), pH 7.2, and cell extract (4 mg), and the reaction mixture was quenched with acid (final pH ~ 1). CO was added at 180 min. The NMR spectra were recorded at 25 °C and pH 1. The spectrum recorded at 1200 min was 3-fold more dilute, resulting in a 3-fold higher concentration of methanol and a 3-fold lower signal/noise than the others. Peaks: a, syringate; b, methanol internal standard; c, CH_3-H_4folate; d, acetyl-CoA; e, acetate; f, acetyl phosphate. (b) (i) Concentrations of syringate (●), CH_3-H_4folate (△), and acetyl-CoA (□) as a function of time. (ii) Concentrations of hydroxyvanillate (○), acetate (◇), and acetyl phosphate (▽) as a function of time.

A set of experiments were performed in which CO was present from the outset. First, the components required for acetyl-CoA formation (measured by NMR) were established (Table 2). A strict requirement for H_4folate, ATP, CoA, and CO was observed. A requirement for CODH, C/Fe-SP, and MeTr was also indicated. The reason that there was only a stimulation of the reaction by addition of these purified proteins instead of a complete dependence is that they were present in the cell extract. In Figure 5, the time course of the O-demethylation reaction is shown. When all of these components were present, the rates of syringate depletion and CH_3-H_4folate formation were the same as those observed in the absence of CO (Figure 4), 0.02 min^{-1}. After 150 min of reaction in the presence of CO, conversion of CH_3-H_4folate to acetyl-CoA, acetyl phosphate, and acetate was observed (data not shown).

When Ti(III) citrate or H_2 was used as reductant, CH_3-H_4folate formation mirrored syringate depletion and the rate of O-demethylation was the same as above (0.02 min^{-1}) (data not shown). In the presence of both Ti(III) and CO, acetyl-CoA was not formed, indicating that Ti(III) inhibits the conversion of CH_3-H_4folate, CO, and CoA to acetyl-CoA.

Effect of Propyl Iodide on the Reaction. Cell extracts were incubated with propyl iodide for 15 min before the reaction was started with syringic acid. In these experiments, syringate was in excess relative to H_4folate. In both the spectrophotometric assay (data not shown) and the TLC determination (Figure 6, lanes 1 and 2), there was no inhibition

Table 2: Components Required for Acetate Formation from Syringate

reaction mixture	acetate formed[a] (% of complete plus CO)
complete plus CO	100
complete minus CO	0
minus H_4folate	3
minus ATP	3.5
minus CoA	5.0
minus C/Fe-SP	63
minus CODH	61
minus MeTr	48
minus MeTr, C/Fe-SP, CODH	38
100-fold reduced CODH	73
50-fold reduced CODH	83
10-fold reduced CODH	95

[a] Measured by ^{13}C-NMR.

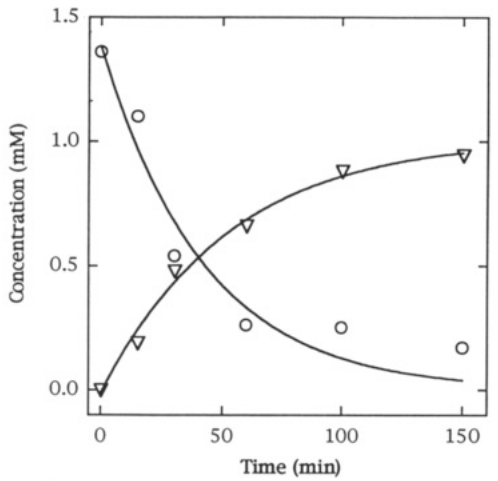

FIGURE 5: ^{13}C-NMR study of the O-demethylation of syringate in the presence of CO. Concentrations of syringate (○) and CH_3-H_4-folate (▽) as a function of time. The NMR spectra were recorded at pH ~ 1 at a temperature of 50 °C.

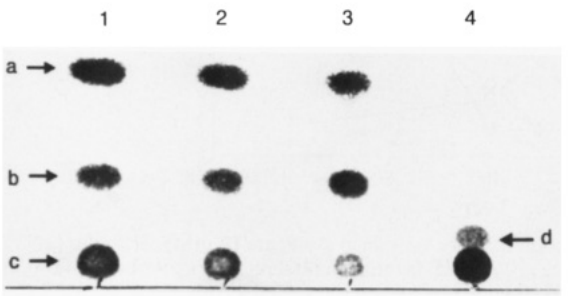

FIGURE 6: Thin-layer chromatography of O-demethylation of syringate (5 mM) in the presence of H_4folate (3.5 mM), ATP (2.5 mM), $MgCl_2$ (2.5 mM), CoA (2.5 mM), CODH (0.15 mg), MeTr (0.1 mg), C/Fe-SP (0.2 mg), and cell extract (1 mg). The reaction was performed in the presence of 1 atm of CO (1.0 mM) and analyzed after 2 h with (lane 1) or without (lane 2) 0.2 M propyl iodide and after 20 h of reaction with (lane 3) or without (lane 4) 0.2 M propyl iodide. The arrows designate (a) syringate, (b) hydroxyvanillate, (c) gallate and (d) pyrogallol.

by propyl iodide of the initial rate of O-demethylation. These results indicate that the O-demethylase is not a corrinoid protein. When the reaction time was extended to 20 h, syringate and hydroxyvanillate were completely converted to gallate and pyrogallol in the absence of propyl iodide (lane 4). However, in the presence of propyl iodide, syringate and hydroxyvanillate were still present after 20 h of reaction time (lane 3). Under these conditions, H_4folate became limiting since the reaction mixture contained 10 mM methyl groups in syringate and only 3.5 mM H_4folate. These results indicate that propyl iodide did not inhibit the O-demethylase but inhibited enzymes involved in the regeneration of H_4folate.

DISCUSSION

Conversion of the methyl group of methoxylated aromatics into the methyl group of acetate is an important reaction in the natural biodegradation of phenyl methyl ethers. One of the unanswered questions has been, where in the pathway does the methyl group enter? This was controversial since H_4folate stimulated the O-demethylation of phenyl methyl ethers by *A. woodii* (Berman & Frazer, 1992) but inhibited acetate formation from vanillate by *S. sucromutans* (Doré & Bryant, 1990). To explain this discrepancy, Kreft and Schink (Kreft & Schink, 1993) proposed that H_4folate does not play a role in the formation of acetyl-CoA from methoxy groups but diverts methyl flux from acetyl-CoA formation.

Our results are consistent with the scheme shown in Figure 1. An assay was developed that included the purified acetyl-CoA pathway enzymes CODH, C/Fe-SP, and MeTr. It was expected that if one or more enzymes were involved in converting the aromatic *O*-methyl group to the methyl group of acetate, their addition to the assay mixture would increase the reaction rate. Instead of complete dependence, a stimulation was anticipated since cells grown on syringate still have relatively high levels of acetyl-CoA pathway enzymes. By determining the dependence of activity on each of these enzymes, one could infer at which step in the pathway the methyl group entered. For example, if the methyl group were transferred directly to CODH, then it would be expected that addition of CODH would increase the rate of acetyl-CoA formation from syringate, but addition of H_4folate, C/Fe-SP, and MeTr would not be stimulatory. A requirement for H_4-folate in O-demethylation by *A. woodii* was observed earlier (Berman & Frazer, 1992); however, in these studies, the product of the reaction was not identified. Thus, it was possible that, in these reactions, CH_3-H_4folate was oxidized by the H_4folate pathway enzymes instead of being converted to acetyl-CoA. The results in Table 2 demonstrate that H_4folate is required for acetyl-CoA formation, indicating that the methyl group enters the acetyl-CoA pathway as CH_3-H_4folate. The requirement for MeTr, CODH, and C/Fe-SP is consistent with this interpretation since MeTr catalyzes the transfer of the methyl group of CH_3-H_4folate to C/Fe-SP and CODH catalyzes the conversion of the methylated C/Fe-SP, CO, and CoA to acetyl-CoA (Ragsdale, 1991). ATP also was required, as found before (Berman & Frazer, 1992). Since ATP is not required for the synthesis of acetyl-CoA from CH_3-H_4folate, it is required for the O-demethylase reaction, although its role remains undefined.

CH_3-H_4folate recently was shown to be formed when H_4-folate and phenyl ethyl ethers were provided to extracts of *Sporomusa ovata* (Stupperich & Konle, 1993) and a methyl chloride utilizing acetogenic strain MC (Messmer *et al.*, 1993). We used NMR spectroscopy to corroborate that the aromatic *O*-methyl group enters the acetyl-CoA pathway as CH_3-H_4-folate and to determine whether H_4folate is the initial methyl acceptor or one or more methyl-transfer reactions precede the formation of CH_3-H_4folate. By following the metabolism of syringate by ^{13}C-NMR spectroscopy, it was possible to identify and quantitate the substrates, stable intermediates, and products of the O-demethylation reaction. Under all conditions examined, the rate of formation of CH_3-H_4folate was equal to the rate of depletion of syringate. In the absence of CO, CH_3-H_4folate accumulated as a stable product. When CO was added, $^{13}CH_3$-H_4folate was converted to [2-^{13}C]acetyl-

CoA, [2-^{13}C]acetyl phosphate, and [2-^{13}C]acetate. Phosphotransacetylase is responsible for conversion of acetyl-CoA to acetyl phosphate, and acetate kinase, for conversion of acetyl phosphate to acetate. The measured rate of acetyl-CoA formation from CH_3-H_4folate, CO, and CoA was ~150 nmol min^{-1} (mg^{-1} of CODH)$^{-1}$, similar to values measured earlier.[1] Thus, the combined results unambiguously demonstrate that the O-demethylase from *C. thermoaceticum* catalyzes transfer of the methyl group of syringate to the N-5 of CH_3-H_4folate as described by eq 1.

$$H_4\text{folate} + \text{Ar-O-CH}_3 \xrightarrow[\text{ATP}]{\text{O-demethylase}} \text{Ar-OH} + CH_3\text{-}H_4\text{folate} \quad (1)$$

A convenient spectrophotometric assay for the O-demethylase involves incubating the reaction mixture with Ti(III) and following the absorbance at 450 nm of the Ti–product complex. The measured rate was the same as that determined by NMR spectroscopy. Since Ti(III) is a strong reductant, it was important to determine whether the O-demethylase underwent or required reductive activation during the assay. A requirement for reductive activation has been well established for other enzymes involved in acetyl-CoA synthesis by *C. thermoaceticum* (Harder *et al.*, 1989; Lu *et al.*, 1990; Lu & Ragsdale, 1991). The O-demethylase from TMBS 4 (Kreft & Schink, 1993) required reduction for activity; however, addition of reductants had no affect on the activity of the O-demethylase in cell extracts of *A. woodii* (Berman & Frazer, 1992). Our results demonstrate that the O-demethylase from *C. thermoaceticum* does not require reductive activation. In addition, the activity was not decreased upon exposure of cell extracts to oxygen. The rates of O-demethylation of syringate were approximately the same whether CO (E_0' at pH 7.0 = −540 mV), Ti(III) citrate (E_0' at pH 7.0 = −480 mV) (Zehnder & Wuhrmann, 1976), or H_2 (E_0' at pH 7.0 = −420 mV) was present. The same rate of demethylation was also observed in reaction mixtures poised at a redox potential of −340 mV or in the total absence of any reductants. Although Ti(III) has no effect on the O-demethylase from *C. thermoaceticum*, it was found to inhibit one of the reactions involved in the conversion of CH_3-H_4folate, CO, and CoA to acetyl-CoA.

Propyl iodide is an inhibitor of methionine synthase (Taylor, 1982) and other corrinoid-dependent methyltransferases. Demethylation of syringate by strain TMBS 4 in the presence of CO_2 was found to be inhibited by propyl iodide in a manner that was reversible by light (Kreft & Schink, 1993). Propyl iodide also inhibited methoxybenzoate utilization by *S. ovata* (Stupperich & Konle, 1993). Therefore, it was proposed that demethylation by TMBS 4 and *Sporomusa* occurred by a corrinoid enzyme. However, with *C. thermoaceticum* cell extracts, the initial rate of O-demethylation of syringate to CH_3-H_4folate was unaffected by propyl iodide at concentrations as high as 0.2 M. These results combined with the lack of a requirement for reductive activation indicate that the O-demethylase from *C. thermoaceticum* is not a corrinoid protein. Although the initial velocity is unchanged, we observed an effect of propyl iodide on the amount of product formed at the end of the reaction. In the presence of CO and in the absence of propyl iodide, CH_3-H_4folate is converted to acetate, and H_4folate is regenerated. However, in the presence of propyl iodide, syringate cannot be fully demethylated and hydroxyvanillate accumulates. We suggest that the observed inhibition is due to propylation of CODH (Ragsdale & Wood, 1985) or of C/Fe-SP. In the presence of propyl iodide, regeneration of H_4folate could not occur and O-demethylation would cease when the H_4folate was completely converted to CH_3-H_4folate, causing inhibition of gallate formation.

Besides the conversion of the methyl group of phenyl methyl ethers to acetyl-CoA, other fates are possible. For example, with strain TMBS 4, in the presence of sodium sulfide, the methyl group can be converted to methane thiol; in the presence of CO_2, it is converted to acetate. Since CH_3-H_4folate is the product of O-demethylation of phenyl methyl ethers, it was expected that under appropriate conditions CH_3-H_4folate should undergo oxidation by the H_4folate pathway to methylene-, methenyl-, and formyl-H_4folate and formate. Formate could then be oxidized to CO_2. This possibility was supported by the observation that addition of reductants or CoA had no affect on the activity of the O-demethylase in cell extracts of *A. woodii* (Berman & Frazer, 1992). With cell extracts of *C. thermoaceticum*, in a reaction mixture containing excess H_4folate but lacking CO, CH_3-H_4folate accumulated as a stable product and none of the oxidation products just mentioned were detected. Conversion of CH_3-H_4folate to CO_2 is thought to provide the energy for growth on phenyl methyl ethers. Although we have not yet focused on this aspect of the problem, the inability to observe ^{13}C-labeled oxidation products may be due to the low levels of the necessary oxidized cofactors (NAD, NADP, etc.) in the reaction mixture.

The O-demethylase from *C. thermoaceticum* was found to have a broad substrate specificity. Besides syringic and hydroxyvanillic acids, acetosyringone and dicamba were also demethoxylated. The nonspecificity and oxygen insensitivity of this enzyme may be useful in biotechnological applications, such as the exploitation of anaerobes for bioremediation of aromatic compounds and the development of plants which are tolerant to treatment with methoxylated aromatic herbicides.

[1] At pH 5.5, the rate of conversion of CH_3-H_4folate, CO, and CoA to acetyl-CoA is ~800 nmol min^{-1} (mg of CODH)$^{-1}$, and at the pH value in these experiments (pH 7.2), it is approximately 160 nmol min^{-1} (mg of CODH)$^{-1}$ (Roberts *et al.*, 1992). The specific activity of the O-demethylase in the cell extract was found to be 15 nmol min^{-1} (mg of cell protein)$^{-1}$, similar to the values determined earlier (Berman & Frazer, 1992; Wu *et al.*, 1988). Since in the ^{13}C-NMR experiments we used 1 mg of purified CODH and 10 mg of cell protein, the rates of CH_3-H_4folate formation and conversion of CH_3-H_4folate, CO, and CoA to acetyl-CoA would be expected to be approximately equivalent (~150 nmol min^{-1}).

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