Demethylation and degradation of phenylmethylethers by the sulfide-methylating homoacetogenic bacterium strain TMBS 4

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Abstract. Biochemical studies on anaerobic phenylmethylether cleavage by homoacetogenic bacteria have been hampered so far by the complexity of the reaction chain involving methyl transfer to acetyl-CoA synthase and subsequent methyl group carbonylation to acetyl-CoA. Strain TMBS 4 differs from other demethylating homoacetogenic bacteria in using sulfide as a methyl acceptor, thereby forming methanethiol and dimethylsulfide. Growing and resting cells of strain TMBS 4 used alternatively CO_2 as a precursor of the methyl acceptor CO for homoacetogenic acetate formation. Demethylation was inhibited by propyl iodide and reactivated by light, indicating involvement of a corrinoid-dependent methyltransferase. Strain TMBS 4 contained ca. 750 nmol g dry mass⁻¹ of a corrinoid tentatively identified as 5-hydroxybenzimidazolyl cobamide. A photometric assay for measuring the demethylation activity in cell extracts was developed based on the formation of a yellow complex of Ti^{3+} with 5-hydroxyvanillate produced from syringate by demethylation. In cell extracts, the methyltransfer reaction from methoxylated aromatic compounds to sulfide or methanethiol depended on reductive activation by Ti^{3+} . ATP and Mg^{2+} together greatly stimulated this reductive activation without being necessary for the demethylation reaction itself. The specific activity of the transmethylation enzyme system increased proportionally with protein concentration up to 3 mg ml⁻¹ reaching a constant level of 20 nmol min⁻¹ mg⁻¹ at protein concentrations ≥ 10 mg ml⁻¹. The specific rate of activation increased in a non-linear manner with protein concentration. Strain TMBS 4 degraded gallate, the product of sequential demethylations, to 3 acetate through the phloroglucinol pathway as found earlier with *Pelobacter acidigallici*.

Key words: Methyl transfer — Demethylation — Methoxylated aromatic compounds — Ether cleavage — Corrinoids — Homoacetogenic bacteria — Phloroglucinol pathway — Dimethylsulfide

Aerobic degradation of ether compounds is a well-known process (Axelrod 1956). Hydroxylation of a carbon atom vicinal to the ether bridge by a monooxygenase reaction transforms the stable ether bond into a readily decomposing hemiacetal structure (Bernhardt et al. 1970). Anaerobic cleavage of ether linkages was first demonstrated in methanogenic enrichment cultures (Healy and Young 1979), and pure cultures of homoacetogenic bacteria demethylating methoxylated aromatic compounds were isolated later (Bache and Pfennig 1981). Numerous other bacteria, predominantly homoacetogens, capable of anaerobic ether cleavage have since been described (Schink et al. 1992).

Studies on the biochemistry of this ether cleavage reaction with ^{18}O -labeled phenylmethylethers demonstrated that the oxygen stays attached to the aromatic residue, and the cleavage is a demethylation rather than a hydrolytic demethoxylation reaction (DeWeerd et al. 1988). Experiments with cell extracts were only of limited success (Wu et al. 1988; Doré and Bryant 1990), probably due to the complexity of the methyltransfer sequence from the substrate to acetyl-CoA synthase, and subsequent carbonylation to acetate. Berman and Frazer (1992) obtained higher demethylation activities in cell extracts by using H_4 folate as methyl acceptor, thus circumventing the steps of acetate synthesis.

The recently isolated strain TMBS 4 uses sulfide as methyl acceptor (Bak et al. 1992). Methylation of sulfide is a property not shared by known homoacetogenic bacteria, but widespread in sediment ecosystems (Finster et al. 1990). In sediments, bacteria similar to strain TMBS 4 outnumber other homoacetogens, but cannot be isolated by enrichment procedures which select for faster growing organisms similar to *Acetobacterium woodii* (Bak et al. 1992).

Abbreviations: BV, benzyl viologen; CTAB, cetyltrimethylammonium bromide; H_4 folate, tetrahydrofolate; MOPS, 3-[N-morpholino]propanesulfonic acid; MV, methyl viologen; NTA, nitrilotriacetate; t_d , doubling time; TMB, 3,4,5-trimethoxybenzoate

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Methyltransfer from methylethers to sulfide can be expected to be an even simpler system than transfer to H_4 folate, if sulfide methylation is a side reaction of the demethylating methyltransferase with sulfide, rather than being catalysed by a special methyltransferase. Hence, we began studying the demethylation reaction in cell suspensions and cell extracts of strain TMBS 4.

Materials and methods

Media and growth conditions

Strain TMBS 4 (DSM 6591) was kindly provided by Dr. F. Bak, Marburg, Germany. Strain TMBS 4 was grown in bicarbonate-buffered (pH 7.2) freshwater mineral medium prepared as previously described, under a N_2/CO_2 (9:1, v/v) atmosphere (Widdel and Pfennig 1981). It contained 8-vitamin solution (Finster et al. 1992); trace element solution SL 10 (Widdel et al. 1983); Na-thiosulfate (50 μM) as sulfur source, and Na-ascorbate or cysteine $\cdot$ HCl (1 mM) as reducing agent. Substrates (1–6 mM) were added as Na-salts to the medium from filter-sterilized anoxic stock solutions. Cultures were incubated at 30 °C in the dark.

Growth parameters at various substrate concentrations (1–6 mM) were determined measuring optical density (578 nm, 1 cm light path cuvettes), substrate and acetate concentrations at regular time intervals. Cell dry mass was determined directly at the end of growth by filtration of the culture. In the case of gallate, the optical densities were corrected for the gallate concentration-dependent bluish color of the medium.

Preparation of cell suspensions and cell extracts

All steps were performed under N_2 atmosphere in rubber-sealed glass vessels (120 ml, 25 ml, 8 ml, or 4 ml) which were kept on ice. Cultures, suspensions, or extracts were transferred between vessels using gas-tight tube connections or syringes. Cells were harvested at the late exponential growth phase by centrifugation at $2000 \times g$ for 20 min, washed once with anoxic 50 mM potassium phosphate or MOPS/ K^+ buffer, pH 7.2, and resuspended in the same buffer. All cell suspension experiments were performed anoxically at room temperature in aluminum-wrapped glass vials. Activities were expressed on the basis of protein concentration. For light-treatment of cell suspensions (2 ml of cell suspension with an OD_{578} of 5 in glass vials with 1.2 cm i.d.) light from a cold light halogen lamp (150 W) was used. The vial was exposed to a light intensity of $1400 \mu\text{mol s}^{-1} \text{ m}^{-2}$, measured with a Li-189 (LI-COR, Lincoln, Nebraska, USA) quantummeter with quantum sensor.

Cell extracts were prepared by disruption at 140 MPa in a French pressure cell (5 passages) gassed with N_2 . The crude extract was centrifuged at $4300 \times g$ for 30 min to remove cell debris.

Cell extracts with high protein content (ca. 30 mg ml^{-1}) were prepared for methyltransfer assays by a modified procedure: 10 l of culture were harvested in a centrifuge equipped with a continuous flow rotor gassed with N_2 and rinsed with anoxic Ti^{3+} -reduced buffer before use. Cells were transferred into stainless-steel bottles in an anaerobic chamber. The resuspending buffer contained 1 mM MgCl_2 and 1 μg DNase I per mg dry weight. The cell extract was centrifuged at $40000 \times g$ for 30 min in sealed stainless-steel tubes, and aliquots frozen in liquid N_2 .

Determination of enzyme activities

All enzyme activities were measured anoxically at room temperature. Gallate decarboxylase was assayed photometrically after Brune and Schink (1992) with Mg^{2+} -free buffer, 1,2,3,5-Tetrahydroxybenzene: pyrogallol hydroxyltransferase was assayed by discontinuous HPLC analysis according to Brune and Schink (1990) with 1 mM substrate concentrations. Phloroglucinol reductase was

measured photometrically after Whittle et al. (1976). 3-Hydroxy-5-oxohexanoate dehydrogenase was assayed photometrically according to Brune and Schink (1992). Acetyl-CoA synthase was measured with BV after Diekert and Thauer (1978). Hydrogenase was measured after Schink (1985).

The demethylating enzyme system was assayed as methyltransfer activity from the donors TMB or syringate to the acceptors sulfide or methanethiol at room temperature under N_2 gas phase either photometrically or by discontinuous HPLC analysis of conversion of the methoxylated aromatic compounds. Reaction mixtures usually contained 50 mM potassium phosphate or MOPS/ K^+ buffer, pH 7.2, 2 mM titanium(III) plus 3 mM NTA, 2–10 mM TMB or syringate (Na-salts), excess amounts (5 μmol) of the volatile methyl acceptors Na_2S or CH_3SNa , and 1–15 mg protein ml^{-1} in a final volume of 250 μl . The photometric assay was performed in stoppered 5 mm light path cuvettes (770 μl volume) to lower extinction due to high protein concentrations, using a modified cuvette holder. The discontinuous HPLC assay was performed in stoppered Durham tubes (500 μl volume) or larger glass vials.

Corrinoid isolation and purification

A cyanocorrinoid was extracted from cells of a 1 l culture according to Kengen et al. (1988) and purified by HPLC after Stupperich et al. (1986).

Chemical analyses

Aromatic compounds were analysed by C_{18} reversed phase HPLC as previously described (Brune and Schink 1990). Samples (100 μl) were injected into 400 μl 100 mM H_3PO_4 and frozen. Acetate was analysed by gas chromatography as previously described (Platen and Schink 1987). CO_2 was measured by injecting headspace samples (300 μl) into a gas chromatograph (Vega 6000, Carlo Erba, Milano, Italy) equipped with a hot wire detector (filament temperature 280 °C), a Carbosieve SII column (100/120 mesh, $2 \text{ m} \times 2 \text{ mm}$, Supelco, Bellefonte, Pa, USA) and an integrator. Conditions were: column, injector and detector temperatures 180 °C, He gas flow 50 ml min^{-1} . For quantification of dimethylsulfide, headspace samples (100 μl) were injected into a gas chromatograph (Vega 6000, Carlo Erba, Milano, Italy) equipped with a flame ionization detector, a Chromosorb 101 column (100/200 mesh, $1 \text{ m} \times 2 \text{ mm}$, Manville, Denver, Co., USA) and an integrator. Conditions were: column temperature 135 °C, injector and detector temperatures 180 °C, N_2 gas flow 40 ml min^{-1} . Standards were prepared by injecting liquid dimethylsulfide into bottles heated in a waterbath to 50 °C (boiling point of dimethylsulfide: 37 °C). A one-step dilution by transfer of gas into a second heated bottle with a syringe followed. Concentrations were calculated using solubility data (Dacey et al. 1984). Protein was determined according to Read and Northcote (1981) using ovalbumin as standard.

Chemicals

Titanium(III)-nitritotriacetate stock solutions were prepared after Moench and Zeikus (1983), with 100 mM TiCl_3 in 150 to 400 mM NTA. The 100 mM Ti^{3+} , 150 mM NTA^{2-} solution, pH 7.0, contained 570 mM NaCl. Triacetic acid (3,5-dioxohexanoic acid) and 1,2,3,5-tetrahydroxybenzene were prepared as described in Brune and Schink (1992) and Brune and Schink (1990), respectively.

All chemicals and gases were of the highest purity available and from standard commercial sources. 5-Hydroxyvanillate was purchased from Pfaltz and Bauer (Waterbury, Conn, USA).

Results

Growth

Strain TMBS 4 grew in sulfide-free media reduced with ascorbate (1 mM) or cysteine (1 mM) with TMB or

gallate as substrate. Growth in both media required addition of thiosulfate (50 μM) as sulfur source. No growth was observed on the reducing agents alone.

Growth yields on gallate and TMB in ascorbate-reduced medium were 8.2 g dry mass mol gallate^{-1} and 22.2 g dry mass mol TMB^{-1} , respectively. Thus, the growth yield per methoxyl group fermented was 4.7 g dry mass mol^{-1} . The growth rate on gallate in this medium was 0.059 h^{-1} ($t_d = 12$ h). Growth rates on TMB were lower and depended on the substrate concentration. At an initial substrate concentration of 4.3 mM or 5.3 mM, growth rates were 0.023 h^{-1} ($t_d = 30$ h) or 0.0195 h^{-1} ($t_d = 35.5$ h), respectively. In fed-batch culture with three subsequent additions of ≤ 2 mM TMB during growth, a higher growth rate of 0.035 h^{-1} ($t_d = 20$ h) was observed in cysteine-reduced medium.

Enzymes of the phloroglucinol and the acetyl-CoA pathway

TMB degradation by cell extracts led to transient formation of syringate and 5-hydroxyvanillate, and syringate degradation to transient formation of 5-hydroxyvanillate, suggesting sequential demethylation of TMB. Table 1 shows activities of enzymes of the phloroglucinol and the acetyl-CoA pathway. Gallate decarboxylase activity was not stimulated by addition of MgCl_2 (1–100 mM) nor did addition of EDTA (1–2.5 mM) inhibit the enzyme. The activity of this enzyme was rather stable in Mg^{2+} -free buffer at 4 $^{\circ}\text{C}$, and decreased to 87% of the initial activity in 17 h.

Table 1. Specific activities of enzymes of the phloroglucinol and the acetyl-CoA pathway

Enzyme	Specific activity [$\text{nmol min}^{-1} \text{mg protein}^{-1}$] in extracts of cells grown on:	
	TMB ^a	gallate ^b
Gallate decarboxylase	120	n.d. ^c
1,2,3,5-Tetrahydroxybenzene: pyrogallol hydroxyltransferase	290	n.d.
Phloroglucinol reductase (NADPH) ^d	n.d.	3300
3-Hydroxy-5-oxo-hexanoate dehydrogenase (NADP ⁺) ^e	4900	n.d.
Acetyl-CoA synthase (BV)	230	1.6
Hydrogenase (MV)	120	1900 ^f

^a Cells were grown on TMB, in vivo activity of TMB degradation was 35 $\text{nmol min}^{-1} \text{mg protein}^{-1}$ (if $t_d = 30$ h), except for acetyl-CoA synthase assay for which cells were grown on syringate

^b In vivo activity of gallate degradation was 240 $\text{nmol min}^{-1} \text{mg protein}^{-1}$

^c not determined

^d Activity with NADH was about 0.2% of that with NADPH as cosubstrate

^e Activity with NAD^+ was 11% of that with NADP^+ as cosubstrate

^f Measured with CTAB permeabilized cells

Methyltransfer in cell suspensions

Demethylation in cell suspensions required either one of the methyl acceptors HS^- , CO, or the CO-precursor CO_2 and was detected only with cells grown on methoxylated aromatic compounds. Demethylation activity increased proportionally with the concentration of either one of the methyl acceptors Na_2S or CO_2 in the concentration range tested (0–2 mM). The rate of TMB degradation with 0.5 mM CO_2 was 8.2 $\text{nmol min}^{-1} \text{mg protein}^{-1}$ and the rate of dimethylsulfide production from syringate with 0.5 mM Na_2S was 6.2 $\text{nmol min}^{-1} \text{mg protein}^{-1}$. CO_2 -dependent demethylation did not require addition of external electron donors. Demethylation activity with 0.5 mM CO as methyl acceptor was 10.5 $\text{nmol min}^{-1} \text{mg protein}^{-1}$. Syringate-grown cells demethylated also TMB and vice versa. Sulfide was used as a methyl acceptor by cells grown in medium without added sulfide. Cells of strain TMBS 4 lost the ability to use CO_2 as methyl acceptor if they were not harvested under strictly anoxic conditions.

Cell suspensions using CO_2 as methyl acceptor formed more than 80% of the amounts of acetate expected for TMB degradation (0.75 acetate per methoxyl group + 3 acetate per aromatic nucleus). Cells growing with TMB formed 96% of the expected amount of acetate.

CO_2 -dependent demethylation of TMB in cell suspensions was inhibited by propyl iodide (Fig. 1). Light treatment of cell suspensions for 5 min revived the activity from 12% to 75% of non-inhibited activity and from 8% to 57% of non-inhibited activity at 50 and 100 μM propyl iodide, respectively. Light had no effect on activity in controls without propyl iodide. Degradation of gallate was not affected by 500 μM propyl iodide. BV-dependent CO oxidation in cell extracts decreased to 67% of uninhibited activity after addition of enough propyl iodide to saturate the reaction mixture (solubility of propyl iodide is ca. 18 mM). Obviously, the strong

Fig. 1. Inhibition of CO_2 -dependent TMB demethylation in cell suspensions by propyl iodide. Cell suspensions (ca. 61 $\mu\text{g protein ml}^{-1}$) were supplied with 30 mM NaHCO_3 , and various amounts of propyl iodide were added from a freshly prepared 10 mM stock solution 10 min before the reaction was started by addition of 2 mM TMB (Na-salt). The activity of the control (0 μM propyl iodide) was 19 $\text{nmol min}^{-1} \text{mg protein}^{-1}$

inhibition of methyltransfer is an effect specific for this activity.

Corrinoid isolation

Cyanide extraction of cells grown on TMB in medium not supplemented with any corrinoid led to isolation of one kind of corrinoid. The purified cyano-corrinoid had the same retention time (16 min) in HPLC at the conditions specified and the same absorption spectrum as authentic $\text{Co}\alpha\text{-}[\alpha\text{-(5-hydroxybenzimidazolyl)]\text{-Co}\beta\text{-cyanocobamide}$ (factor III). Using cyanocobalamin as a standard, a corrinoid content of $750 \text{ nmol g dry mass}^{-1}$ was estimated for strain TMBS 4.

Methyltransfer in cell extracts

Photometric assay of methyltransfer in cell extracts. TMB and syringate were demethylated to 5-hydroxyvanillate which forms a yellow complex with Ti^{3+} . However, the downstream metabolites gallate and pyrogallol are also vicinal-diols and form yellow complexes with Ti^{3+} as well. TMB, syringate, and phloroglucinol do not complex Ti^{3+} . Chemical alterations of the aromatic acids by Ti^{3+} were not observed.

The effect of Ti^{3+} , NTA and Mg^{2+} at various concentrations on linearity and slope of extinction-concentration curves (at different wavelengths from 370 to 470 nm) was examined. Typical curves are shown in Figure 2. Linearity of the parabolic curves increased with i) increasing wavelength beyond that of the absorption maximum (about 375 nm); ii) decreasing the proportion of NTA to Ti^{3+} in the Ti^{3+} -NTA reagent; iii) decreasing the concentration of the Ti^{3+} -NTA reagent and iv) decreasing the Mg^{2+} concentration. Sensitivity of the assay for metabolite concentrations increased with decreasing wavelength and with decreasing proportion of NTA to Ti^{3+} in the Ti^{3+} -NTA reagent, and was only slightly influenced by concentrations of the Ti^{3+} -NTA reagent or of Mg^{2+} . Extinction coefficients at 450 nm

Fig. 2. Extinction of 5-hydroxyvanillate- Ti^{3+} -complexes under various conditions in 1 cm light path cuvettes. 2 mM Ti^{3+} plus 3 mM NTA (○), 2 mM Ti^{3+} plus 8 mM NTA (●), both measured at 400 nm. 2 mM Ti^{3+} plus 3 mM NTA (□), 10 mM Ti^{3+} plus 15 mM NTA (◇), both measured at 450 nm

and at reagent concentrations (2 mM Ti^{3+} plus 3 mM NTA in 50 mM MOPS buffer, pH 7.2) assuring sufficient linearity of the response above 0.1 mM aromatic acid (Fig. 2, □) were: 5-Hydroxyvanillate, $2.5 \text{ mM}^{-1} \text{ cm}^{-1}$; gallate, $2.6 \text{ mM}^{-1} \text{ cm}^{-1}$; and pyrogallol, $1.8 \text{ mM}^{-1} \text{ cm}^{-1}$. The photometrically measured reaction curves were linear with time after an initial short phase of substrate conversion if the cell extract was preactivated before reaction start in a reaction mixture containing Ti^{3+} , ATP, and Mg^{2+} but no substrates.

Comparison of discontinuous and photometric assay of methyltransfer activity. Light had no effect on activation or activity of the reaction measured photometrically. Reaction mixtures put into the light path for 10 s 5 min after reaction start and again after 10 min exhibited a reaction progress equal to that of the control which was permanently placed in the light path of the photometer. During photometric measurement of syringate demethylation, reaction mixtures were simultaneously analysed by HPLC. They accumulated 5-hydroxyvanillate and pyrogallol, but no gallate, and usually no phloro-

Fig. 3 A, B. Stimulation of activation of syringate demethylation activity by ATP A) and Mg^{2+} B) in cell extracts. Reaction progress was measured photometrically at 450 nm in 0.5 cm light path cuvettes. All assays contained 50 mM MOPS buffer (pH 7.2), 10 mM Na-syringate, 5 μmol CH_3SNa , 2 mM Ti^{3+} plus 3 mM NTA, and 50 μl cell extract containing 1 mM MgCl_2 and 33 mg protein ml^{-1} in a final volume of 250 μl . The reaction was started by addition of cell extract, arrows indicate later additions. The maximal activities reached were similar (29% standard deviation) in all assays. The reaction mixtures and additions (arrowed) were as follows. Curve a: 2 mM ATP, 1 mM MgCl_2 and 5 mM glucose; 15 U hexokinase (in 10 μl) was added during the phase of maximal activity. Curve b: as for curve a; 15 U hexokinase were added earlier during the phase of activation; 13 mM ATP was added later as an internal positive control. Curve c: 1 mM MgCl_2 no ATP, hexokinase, or glucose; later 2 mM ATP was added. Curve d: 1 mM MgCl_2 , no ATP, but 15 U hexokinase and 5 mM glucose; later 2 mM ATP was added, even later 13 mM ATP was added as a positive control. This addition resulted in activation (not shown). Curve e: 1 mM MgCl_2 and 10 mM ATP. Curve f: 10 mM ATP, 10 mM EDTA (Na_2) but no MgCl_2 ; later 15 mM MgCl_2 was added

glucinol. The extent of substrate conversion under conditions of different reaction velocities estimated photometrically was 0.90 ± 0.11 times lower than the extent determined by HPLC. The difference was due to pyrogallol production, which has a lower extinction coefficient than 5-hydroxyvanillate.

Reductive activation of methyltransfer. If the reaction was started by addition of cell extract, activity was observed only after a lag phase (Fig. 3), the extent of which depended on the age of the cell extract. In the absence of the reducing agent Ti^{3+} , no activity was detected by HPLC. Activation in the presence of Ti^{3+} was greatly stimulated by the simultaneous presence of ATP and Mg^{2+} (Fig. 3). ADP stimulated the activation reaction as did ATP, but only in the absence of hexokinase and glucose. AMP had no effect.

Dependence of the activation process and specific activity on protein concentration. Both activity and rate of activation, i.e. velocity and velocity of the increase of the concentration of active enzymes (measured as activity per volume) with time, increased not proportionally but in a more complex manner with increasing protein concentration: Specific activity increased proportionally with protein concentration up to ca. 3 mg ml^{-1} and reached a plateau of maximum specific activity of $20 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ at protein concentrations $\geq 10 \text{ mg ml}^{-1}$ (Fig. 4). The *in vivo* activity of CO_2 -dependent demethylation of ca. $35 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ was of the same order of magnitude. The specific rate of activation, i.e. the maximal activity divided by lag phase length and protein concentration, depended strongly on protein concentration in a non-linear manner.

Fig. 4. Dependence on protein concentration of specific activity (●) and specific rate of activation (○) of syringate demethylation by cell extracts. Reaction mixtures contained 50 mM MOPS buffer (pH 7.2), 10 mM Na-syringate, 5 μmol CH_3SNa , 2 mM Ti^{3+} plus 3 mM NTA, 10 mM ATP (Na_2), 1 mM MgCl_2 , and various amounts of cell extract ($33 \text{ mg protein ml}^{-1}$) in a final volume of 250 μl . For measuring activation rates (○) photometrically (450 nm, 0.5 cm light path), the reaction was started by addition of cell extract. The specific activity was measured and divided by the protein concentration and the length of the lag phase of the reaction. Specific activity was measured by HPLC analysis of 5-hydroxyvanillate formation. The cell extract was preincubated in the reaction mixture (same composition as above) without syringate for $2 \times$ the length of the lag phase determined photometrically under corresponding conditions. Then the reaction was started by addition of syringate.

Further characteristics of methyltransfer. Cell extract (100 μl with a protein concentration of 25 mg ml^{-1}) treated with 10 U of proteinase K for 20 min at room temperature was inactive. Heat-treatment (5 min 80°C) inactivated the cell extract as well. O_2 -treatment (100 μl cell extract was gently shaken with 5 ml air for 15 min at room temperature followed by flushing with N_2), caused only a slight loss (12%) of activity. Activity could even be measured with cells harvested under air, but activation processes were rather slow and variable. Oxygen did not destroy activity irreversibly; whether oxygen inhibits reversibly cannot be tested with our system containing Ti^{3+} as reducing agent.

Discussion

In the present study, evidence was found for homoacetogenic metabolism of methyl groups derived from methoxylated aromatic compounds: i) CO_2 was used as methyl acceptor for demethylation in growing and resting cells to form 0.75 acetate per methoxyl group, ii) demethylation supported growth with a yield of 4.7 g dry mass per mol methoxyl group, and iii) acetyl-CoA synthase activity was present in cells grown with methoxylated aromatic compounds. The use of CO_2 as methyl acceptor involves reduction of CO_2 to CO and subsequent synthesis of acetyl-CoA by acetyl-CoA synthase. Strain TMBS 4 did not require external electron donors for CO_2 reduction but obtained those electrons in partial oxidation of methyl groups to CO_2 . Gallate, the product of sequential demethylation of TMB, was degraded by the enzymes of the phloroglucinol pathway (Schink et al. 1992). Cell extracts of strain TMBS 4 catalysed (as far as tested) the same reactions as *Pelobacter acidigallii* extracts. However, gallate decarboxylase activity and stability were not enhanced by Mg^{2+} , in contrast to the corresponding enzyme in *Pelobacter acidigallii* (Brune and Schink 1992). Hence, strain TMBS 4 combines the catabolic capabilities of homoacetogenic bacteria such as *Acetobacterium woodii* (Diekert 1992) and gallate-fermenting anaerobes such as *Pelobacter acidigallii*. The pathway of TMB degradation by strain TMBS 4 is depicted in Fig. 5.

Wu et al. (1988) found demethylation in cell suspensions of *Clostridium thermoaceticum* to depend on CO if cells were grown with CO_2 as methyl acceptor. CO_2 did not support a higher demethylation rate than did N_2 , in contrast to our results. CO differs from CO_2 in being not only a more direct methyl acceptor but also a low-potential reductant which can e.g. activate methyltransfer from $\text{CH}_3\text{-H}_4\text{folate}$ via corrinoid/Fe-S protein to acetyl-CoA synthase in *Clostridium thermoaceticum* (Ragsdale et al. 1987). We found a strictly anoxic cell harvest necessary for maintenance of the ability of cells of strain TMBS 4 to use CO_2 as methyl acceptor.

Propyl iodide is a specific inhibitor of most corrinoid-dependent methyltransferases (Taylor 1982; Kengen et al. 1988 and references therein). Light-reversible inhibition of methyltransfer by propyl iodide in strain TMBS 4 suggests involvement of a corrinoid-dependent enzyme

Fig. 5. Catabolism of 3,4,5-trimethoxybenzoate by strain TMBS 4: sequential demethylations followed by steps of the phloroglucinol pathway. Question marks indicate reactions which have not yet been tested

in the methyltransfer sequence from the methoxylated aromatic compound to acetyl-CoA synthase. H₄folate served as a methyl acceptor in hydroferulate demethylation by cell extracts of *Acetobacterium woodii* (Berman and Frazer 1992). However, Doré and Bryant (1990) found that H₄folate inhibited acetate formation from vanillate by *Syntrophococcus sucromutans*. Both results can be explained assuming that H₄folate is not an intermediate in acetate formation from methoxyl groups, but acts as a sink for methyl groups diverting methyl flux away from acetyl-CoA synthase. In methanogens, the methyl group of methanol is transferred via a corrinoid-containing and a further methyltransferase to coenzyme M without involvement of pterins (van der Meijden et al. 1984a). Berman and Frazer (1992) found catalytic amounts (20 µM) of cob(II)alamin to be ineffective as methyl acceptor. This can be expected since, in the absence of CO₂ or CO, stoichiometric amounts of a potential methyl acceptor would be required, and only cob(I)alamin, not cob(II)alamin accepts methyl cations in a manner similar to H₄folate.

Methylation of sulfide by cells of strain TMBS 4 grown on methoxylated aromatic compounds might either be a specific but constitutive activity, or a side reaction of an enzyme of the methyltransfer sequence by which methyl groups are lost for the metabolism. The protein dependence, ATP stimulation of activation, and inactivation by heat and protease treatments clearly demonstrate sulfide methylation to be an enzymatic reaction. Chemical thiol alkylation by alkylcobalamins could be a model for the reaction mechanism. The mechanism of this methylation is known to be a nucleophilic attack of a thiolate anion on the alkyl carbon (Hogenkamp et al. 1985, 1987).

We developed a photometric assay to measure demethylation. This assay offers the advantage of continuous measurement of the non-linear reaction progress curves

with good time resolution, which is particularly valuable for measuring the length of lag phases and rates of activation. Also, it allows convenient and fast activity tests of fractions in enzyme chromatography and in screening for effects of various additions on enzyme activity. However, there are also disadvantages of this photometric assay: Ti³⁺ acts both as reducing agent and as reaction partner in colour formation. Calibration curves were more or less obviously non-linear, and the choice of reagent concentrations is restricted by the requirement for almost linear curves. Ti³⁺, which has to be kept soluble by some complexing agent, is required for colour formation. This complexing agent lowers the concentration of free Mg²⁺ (and other polyvalent cations) and hence the concentration of Mg-ATP in the assay is not well defined. The first product of demethylation must accumulate and complex Ti³⁺. This will be no problem in work with other homoacetogenic bacteria which do not metabolize the demethylated substrate further.

Significantly high demethylation activities in cell extracts were first demonstrated by Berman and Frazer (1992). We obtained reproducible and high activities of methyltransfer only if high protein concentrations, ATP, and a low-potential reductant were present. Berman and Frazer (1992) also used high protein concentration, ATP, and usually employed reductants such as pyruvate (potential reductant in the presence of CoA and cell extract), dithioerythritol, cysteine, and a gas phase containing 3% H₂. Studies of corrinoid-dependent methyltransfer reactions in catabolism demonstrate in general involvement of ATP and low-potential reductants (H₂, reduced ferredoxin, CO, Ti³⁺, in reductive activation (van der Meijden et al. 1984a, b; Ragsdale et al. 1987; Kengen et al. 1988; van de Wijngaard et al. 1991).

The activity of methyltransfer by extracts of strain

TMBS 4 was not proportional to protein concentration but showed a quadratic dependence up to 3 mg ml⁻¹, i. e., the specific activity was proportional to protein concentration. This finding renders statements on specific activities of this enzyme reaction questionable. Berman and Frazer (1992) also found a quadratic dependence of activity in *Acetobacterium woodii* on protein concentration, although not stating this explicitly: a parabola of the form $y = ax^2$ fits well to their data except for the highest protein concentration used. If the reaction rate is limited by the rate of interaction of two components of the cell extract, e.g. two enzymes, two subunits of one dissociable enzyme, or one enzyme and one coenzyme, the reaction rate would be a quadratic function of cell extract concentration. Application of high protein concentrations is a common feature of reports on demethylation or other methyltransfer systems (Doré and Bryant 1990; Kengen et al. 1990; van de Wijngaard et al. 1991; Berman and Frazer 1992).

The situation is further complicated by the complex dependence of the reductive activation process on protein concentration. This indicates involvement of protein-protein interaction in the activation.

We demonstrated that ATP was not necessary for the demethylation reaction itself once the enzyme system was activated: Addition of hexokinase to remove ATP from the reaction mixture (glucose was present) stopped further activation and the level of activity already reached was maintained. Addition of hexokinase to the completely activated system had no effect on activity. If hexokinase and glucose were not present, a slow activation occurred without addition of ATP, especially if fresh cell extract was used. This effect might be due to low levels of remnant ATP in the concentrated cell extract. The stimulation by ADP probably was due to conversion to ATP by nucleoside diphosphate kinase in the cell extract, because hexokinase and glucose prevented the ADP effect. However, nucleoside diphosphate kinase activity was not tested.

Future work in our laboratory will concentrate on elucidation of activity and activation of the methyltransferase system.

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