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Corrinoid-Dependent Methyl Transfer Reactions Are Involved in Methanol and 3,4-Dimethoxybenzoate Metabolism by *Sporomusa ovata*

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Washed and air-oxidized proteins from *Sporomusa ovata* cleaved the C-O bond of methanol or methoxyaromatics and transferred the methyl to DL-tetrahydrofolate. The reactions strictly required a reductive activation by titanium citrate, catalytic amounts of ATP, and the addition of DL-tetrahydrofolate. Methylcorrinoid-containing proteins carried the methanol methyl, which was transferred to DL-tetrahydrofolate at a specific rate of 120 nmol h^{-1} mg of protein^{-1}. Tetrahydrofolate methylation diminished after the addition of 1-iodopropane or when the methyl donor methanol was replaced by 3,4-dimethoxybenzoate. However, whole *Sporomusa* cells utilize the methoxyl groups of 3,4-dimethoxybenzoate as a carbon source by a sequential O demethylation to 4-hydroxy-3-methoxybenzoate and 3,4-dihydroxybenzoate. The in vitro O demethylation of 3,4-[4-*methoxyl*-^{14}C]dimethoxybenzoate proceeded via two distinct corrinoid-containing proteins to form 5-[^{14}C]methyl-tetrahydrofolate at a specific rate of 200 nmol h^{-1} mg of protein^{-1}. Proteins from 3,4-dimethoxybenzoate-grown cells efficiently used methoxybenzoates with vicinal substituents only, but they were unable to activate methanol. These results emphasized that specific enzymes are involved in methanol activation as well as in the activation of various methoxybenzoates and that similar corrinoid-dependent methyl transfer pathways are employed in 5-methyl-tetrahydrofolate formation from these substrates. Methyl-tetrahydrofolate could be demethylated by a distinct methyl transferase. That enzyme activity was present in washed and air-oxidized cell extracts from methanol-grown cells and from 3,4-dimethoxybenzoate-grown cells. It used cob(I)alamin as the methyl acceptor in vitro, which was methylated at a rate of 48 nmol min^{-1} mg of protein^{-1} even when ATP was omitted from the assay mixture. This methyl-cob(III)alamin formation made possible a spectrophotometric quantification of the preceding methyl transfers from methanol or methoxybenzoates to DL-tetrahydrofolate.

Sporomusa ovata utilizes the methyl groups of methanol or methoxybenzoates in the formation of acetyl coenzyme A. The metabolism of both substrates requires an anaerobic C-O bond cleavage as the initiating reaction. The mechanistic details of these activities, the methyl carriers, and the methyl acceptors are currently being investigated, since immunological evidence that methanol induces a corrinoid-containing protein in *S. ovata* has been presented (17).

Methanol is a methanogenic substrate for *Methanosarcina barkeri*. The methyl is transferred to thioethanesulfonate (S-CoM) via a corrinoid-containing enzyme (21). The product of this reaction, CH_3-SCoM, is further reduced to methane. However, S-CoM and CH_3-SCoM are specific methanogenic cofactors, which are absent in acetogens, and therefore a different methyl transfer pathway for *Sporomusa* spp. has to be considered.

Several acetogenic (4–6, 9–11, 16, 19, 22) and nonacetogenic (8, 12, 13, 20) bacteria cleave aromatic and aliphatic *O*-methyl ethers under anaerobic conditions. These growth experiments were carried out after the first report on the selective isolation of *Acetobacterium woodii* with methoxybenzoates as carbon sources was published (1). The *O*-methyl ether cleavage mechanisms are O demethylations in *Acetobacterium* and *Eubacterium* spp., because ^{18}O-labeled methoxybenzoates were converted into ^{18}O-labeled hydroxybenzoates (7). In vitro experiments with cell extracts from *A. woodii* also indicated the importance of DL-tetrahydrofolate and ATP in the anaerobic hydroferulate O demethylation, but methyl carriers or methyl acceptors have not yet been identified (4).

This paper reports on the utilization of methanol and 3,4-dimethoxybenzoate by *S. ovata*. The substrates serve as methyl donors in vitro when DL-tetrahydrofolate is present as a methyl acceptor. Protein-bound [*methyl*-^{14}C]methyl-corrinoids and 5-[^{14}C]methyl-tetrahydrofolate were found after the application of *methyl*-^{14}C-labeled substrates. These findings are consistent with corrinoid-dependent methyl transfers from methanol or 3,4-dimethoxybenzoate to DL-tetrahydrofolate.

MATERIALS AND METHODS

Bacteria. *S. ovata* H1 (DSM 2662) was cultivated in mineral salt medium at 28°C (18). Media were supplemented with 50 mM sodium bicarbonate and either 120 mM methanol or 10 mM 3,4-dimethoxybenzoate.

Chemicals. Aromatics, DL-tetrahydrofolate, and 5-methyl-tetrahydrofolate were from Fluka, Neu-Ulm, Germany. 8-Azido-ATP was obtained from ICN Biochemicals. Light-sensitive Co-methyl, Coβ-acetyl and Coβ-propyl derivatives of *p*-cresolyl cob(I)amide (pCC) were prepared in darkness by treatment of pCC with a fivefold excess of methyl iodide, acetic anhydride, or 1-iodopropane, respectively, for 30 min at room temperature. The pCC was formed in 5 mM aqueous titanium citrate, and the Coβ-alkyl corrinoids were separated and purified by reversed-phase C_{18} high-pressure liquid chromatography (RP18-HPLC) (Table 1).

Radiochemicals. Cobalt-57 chloride (specific radioactivity, 3×10^5 Bq/μmol) and [^{14}C]methyl iodide (specific radioac-

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TABLE 1. Separation of corrinoids, tetrahydrofolates, and aromatics by using RP18-HPLC

Compound	Gradient[a]	Detection wavelength (nm)[b]	Retention time (min)
Coβ-OH,Coα-CN-pCC	1	254, 540	19.8
Coβ-CN,Coα-OH-pCC	1	254, 540	25.6
Coβ-OH,Coα-CH_3-pCC	1	254, 540	26.6
Coβ-CH_3,Coα-OH-pCC	1	254, 540	30.8
Coβ-acetyl-pCC	1	254, 540	28.2
Coβ-propyl-pCC	1	254, 540	33.6
Tetrahydrofolate	2	240, 280	10.3
5-Methyl-tetrahydrofolate	2	240, 280	11.5
3,4-Dimethoxybenzoate	3	258	48.0
4-Hydroxy-3-methoxybenzoate	3	258	24.9
3-Hydroxy-4-methoxybenzoate	3	258	30.1
3,4-Hydroxybenzoate	3	258	10.2

[a] The stationary phase was Nucleosil 120-10 C_{18}, and the mobile phases were linear methanol gradients in 17 mM acetic acid, as follows: gradient 1, 23 to 60% methanol within 30 min, followed by 5 min of isocratic 60% methanol; gradient 2, 0 to 45% methanol within 15 min; gradient 3, 18% isocratic methanol within 0 to 35 min, 18 to 40% isocratic methanol within 3 min, and an additional 12 min of isocratic 40% methanol.

[b] Two numbers indicate double-wavelength detection mode.

tivity, 13.6 MBq/mg) were from Amersham Buchler, Braunschweig, Germany. [^{14}C]methanol (specific radioactivity, 125 MBq/mmol) was from NEN. 3,4-[4-*methoxyl*-^{14}C]dimethoxybenzoate was synthesized from 1 mmol of 4-hydroxy-3-methoxybenzoate in 1 ml of 10% KOH and 0.16 mmol of [^{14}C]methyl iodide (specific radioactivity, 230 kBq/μmol). The reaction mixture was stirred for 24 h at 70°C under a nitrogen gas phase. Thereafter, it was acidified to pH 3.5 with glacial acetic acid before the homogeneous 3,4-[4-*methoxyl*-^{14}C]dimethoxybenzoate fraction was isolated from this solution by using a preparative RP18-HPLC column (7.8 mm by 30 cm; Nucleosil 120-10 C_{18}). The retention time of 3,4-[4-*methoxyl*-^{14}C]dimethoxybenzoate was 20 min in 42% methanol–58% 17 mM acetic acid at a flow rate of 2 ml min^{-1}. The HPLC solvent was removed from the labeled product in a rotary evaporator (3 kPa, 35°C). Radioactive carbon compounds and ^{57}Co-labeled corrinoids were quantified in a beta scintillation counter (Kontron BETAmatic) and in a gamma scintillation counter (Berthod Gammaszint BF 5300), respectively.

Preparation of cell extracts. The following procedure was performed in air. Five grams (wet weight) of cells was homogenized in 10 ml of 100 mM Tris-HCl (pH 7.2) before the cells were disrupted in a French press (130 MPa). Cell debris and membranes were separated from soluble proteins by centrifugation (150,000 × *g*, 4°C, 1 h). The supernatant was mixed with 5 mg of protamine sulfate per ml and kept on ice for a 30-min agitation period. Another centrifugation (5,000 × *g*, 4°C, 10 min) pelleted insoluble protamine complexes. Thereafter, 1-ml fractions of that supernatant were each concentrated to ca. 100 μl by using Centricon 30 microconcentrators. The filtration residues were washed twice with 1 ml of 100 mM Tris-HCl (pH 7.2), and in some experiments, one additional wash with 1 ml of 10 mM EDTA was carried out before the filtration residue was resuspended in 1 ml of Tris-HCl buffer. These protein solutions contained ca. 1 mg of protein per 25 μl; they were stored at −20°C until use.

In vitro assay for methyl transfer activities. Methyl transfers were measured in 1-ml quartz cuvettes which contained a nitrogen gas phase and which were sealed by rubber stoppers. The reaction mixture consisted of 86 mM Na-PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.2), 3.5 mM $MgCl_2$, 100 μM ATP, 1 mM DL-tetrahydrofolate, 4 mM titanium citrate, and either 10 mM methanol or 5 mM methoxyaromatics in a total volume of 580 μl. Methyl transfers were started by the addition of 25 μl (ca. 1 mg) of protein solution, and they proceeded routinely for 30 min at 35°C in darkness. Ten-microliter aliquots were withdrawn during this incubation period to quantify the 5-methyltetrahydrofolate formation by means of HPLC analyses. For spectrophotometric quantification of the methyl transfer activities, 170 μM HO-cobalamin was also injected in the assay. An increasing A_{372} was indicative for the production of the essential methyl trap cob(I)alamin by titanium citrate. The reaction product CH_3-cob(III)alamin was quantified from the differences in A_{528} (ε_{528} = 7,900 cm^{-1}/M^{-1}) or, during some experiments, by withdrawing 10-μl assay aliquots and analyzing them for methyl-cobalamin by using HPLC. Otherwise, the methyl transfer reactions were terminated by the injection of 5 mM 1-iodopropane in ethanol. The corrinoid-containing enzymes were then isolated from the assay mixtures by gel permeation chromatography. Separated enzymes were subsequently extracted for their corrinoid cofactors with acetic acid, and again HPLC analyses were used to identify their light-sensitive Coβ ligands.

Analytical methods. (i) HPLC. Corrinoids, tetrahydrofolates, methoxyaromatics, and phenols were separated by RP18-HPLC. The stationary phase was Nucleosil 120-10 C_{18} (3.9 mm by 30 cm; Machery & Nagel, Düren, Germany), and the mobile phase was methanol in 0.1% aqueous acetic acid at a flow rate of 1 ml min^{-1}. The eluate was monitored simultaneously at two wavelengths by means of a variable-wavelength detector (Millipore Waters model 490E). Table 1 compiles the chromatographic conditions, detector settings, and retention times for the particular compounds.

(ii) FPLC. For fast protein liquid chromatography (FPLC), proteins were fractionated by using a gel filtration column (HiLoad Superdex 200 16/60; Pharmacia). The column was operated with 100 mM Tris-HCl (pH 7) at a flow rate of 0.8 ml min^{-1}. Two-milliliter fractions were collected in reagent tubes to determine the enzyme activities and the radioactivities.

(iii) UV-visible spectroscopy. Absorption spectra of aromatics and corrinoids were recorded with a Kontron Uvikon 860 spectrophotometer and quartz cuvettes (path length = 1 cm) in the spectral range from 200 to 650 nm. The aromatic compounds were dissolved in methanol–17 mM acetic acid (1:1), and the corrinoids were prepared in water.

(iv) ^{1}H-NMR. The degradation products of 3,4-dimethoxybenzoate were isolated from acidified culture media (pH 2 to 3) by RP18-HPLC. The HPLC solvent was removed from the aromatics by flash evaporation (4 kPa, 40°C), and the residues were twice dissolved in 2 ml of D_2O and evaporated to dryness. These samples were finally resuspended in D_2O at 5 to 10 mM concentrations before the ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra were recorded with a Bruker MSL 400 instrument at 400.13 MHz and ambient temperature. All ^{1}H-NMR measurements were determined in reference to 4,4-dimethyl-4-silapentane sodium carboxylate in D_2O.

(v) Protein and ATP determinations. Protein concentrations were determined by a modified Lowry protocol (14), and ATP was quantified spectrophotometrically by means of

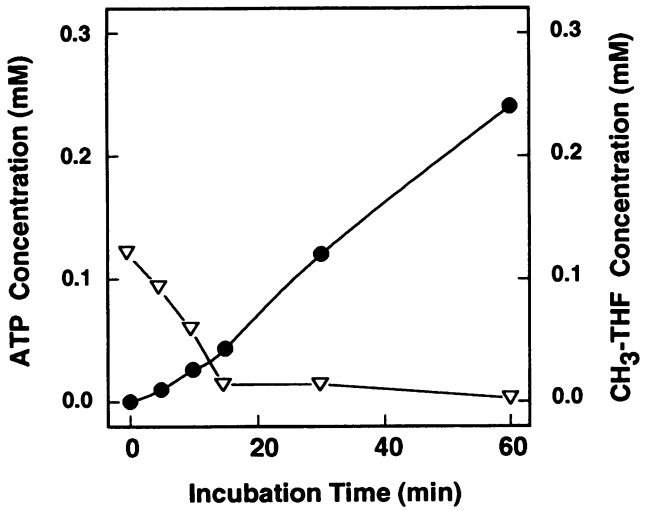

FIG. 1. In vitro 5-methyl-tetrahydrofolate (CH_3-THF) formation from methanol (●) activated by ATP (▽).

D-glucose, hexokinase (EC 2.7.1.1), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (3).

RESULTS

Methyl transfer reactions which are involved in the anaerobic metabolism of methanol and 3,4-dimethoxybenzoate by *S. ovata* were studied. One in vitro assay was established for this purpose. It employed carefully washed proteins in order to avoid disturbing interferences with low-molecular-weight compounds and to provide clear evidence for the in vivo CH_3 acceptors. Spectrophotometric quantifications of these reactions were easily achieved by using cob(I)alamin as an artificial methyl trap. The cob(I)alamin methylation was quantified from spectral changes in its UV-visible spectrum.

Methanol metabolism. Washed proteins from methanol-grown cells used the methanol methyl in the formation of 5-methyl-tetrahydrofolate. The methyl transfers occurred only when the assay mixture was reduced by titanium citrate and when DL-tetrahydrofolate was present. In addition, up to 120 μM ATP was necessary to activate the reactions, which thereafter continued for more than 60 min (Fig. 1). It became evident from the ATP and 5-methyl-tetrahydrofolate concentrations during the course of the experiment that the nucleoside triphosphate was consumed mainly during an initial activation phase. At the end of the experiment, approximately 50 nmol of ATP was used up in the production of 130 nmol of 5-methyl-tetrahydrofolate. This result indicates that up to 3 mol of methyl-tetrahydrofolate per mol of ATP was generated at a reaction rate of 130 nmol min^{-1} mg of $protein^{-1}$. The apparent K_m values for methanol, ATP, and DL-tetrahydrofolate were 2 mM, 320 μM, and 530 μM, respectively.

A comparable reaction acceleration was observed when ATP was replaced by 125 μM 8-azido-ATP in darkness. When a parallel sample, however, was illuminated for 20 min (250 W, 20-cm distance) before the incubation, the overall 5-methyl-tetrahydrofolate formation was reduced to less than 15 nmol h^{-1} mg of $protein^{-1}$.

Two low-molecular-weight compounds were labeled when [^{14}C]methanol was used as the substrate and HO-cobalamin was also added to the assay mixture. One of these compounds cochromatographed with authentic 5-methyl-tetrahydrofolate (data not shown), supporting the assumption that tetrahydrofolate is the methyl acceptor in vivo. The other ^{14}C-labeled low-molecular-weight compound cochromatographed with authentic CH_3-cobalamin. This corrinoid was resistant to cyanolysis in 1 mM KCN in darkness. A 30-min illumination of the corrinoid solution with a 250-W bulb at a 20-cm distance split off the labeled Co-ligands, which were separated thereafter from the nonlabeled corrinoid by RP18-HPLC.

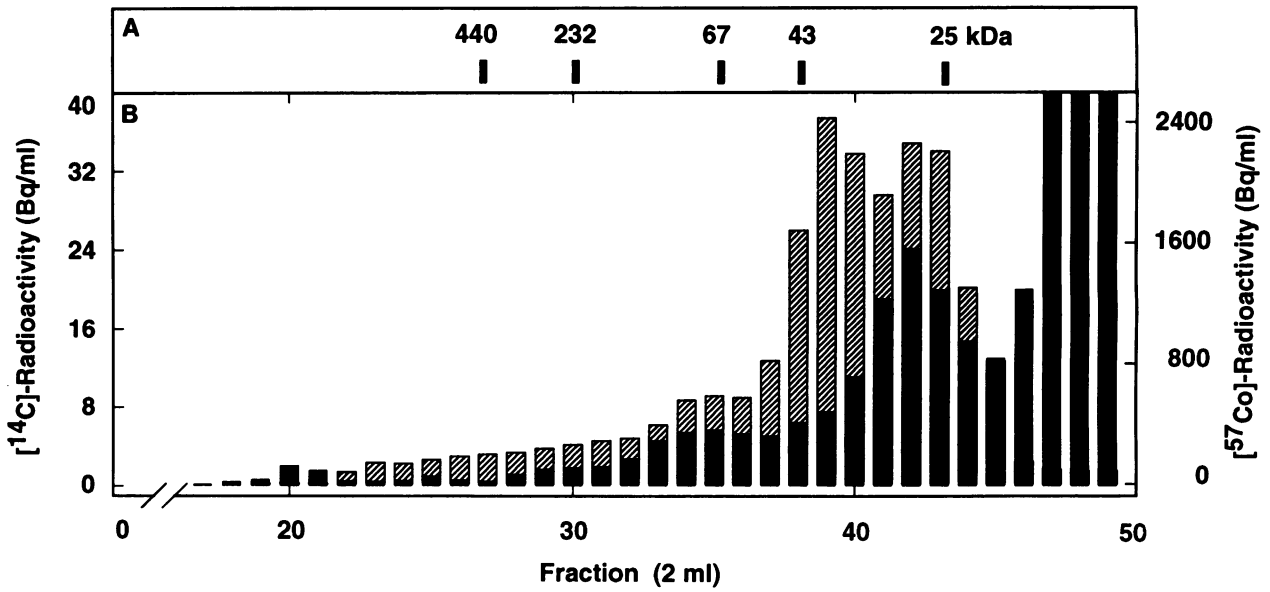

FIG. 2. Gel permeation chromatography of an in vitro assay for 5-methyl-tetrahydrofolate formation from [^{14}C]methanol. The reaction was performed with ^{57}Co-labeled proteins, and it was terminated by the addition of 1-iodopropane. (A) Molecular weight markers; (B) distributions of the ^{14}C-radioactivity (■) and of the ^{57}Co radioactivity (▨).

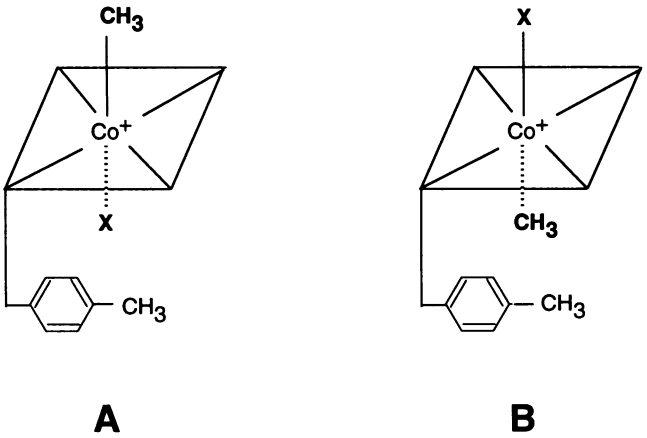

FIG. 3. Cobalt-methyl isomers of pCC. (A) Coβ-methyl-pCC; (B) Coα-methyl-pCC. X, either a water molecule in vitro or a not-yet-identified cobalt ligand in vivo.

The methyl transfers from methanol to DL-tetrahydrofolate and cob(I)alamin were independent of the cell membranes, since soluble proteins with molecular masses of 10 to 400 kDa, as determined by gel permeation chromatography, catalyzed these reactions. The reaction rate was 120 nmol min^{-1} mg of $protein^{-1}$, even when the enzymes were washed with 10 mM EDTA solution prior to the incubation. This finding suggests that readily interchangeable metal ions might not participate in the activation and transfer reactions.

The DL-tetrahydrofolate methylation was inhibited after the injection of 2 mM 1-iodopropane into the assay mixture, suggesting that corrinoid-dependent enzymes could be involved in the methyl transfer from methanol to 5-methyltetrahydrofolate. Indeed, two ^{14}C-containing protein fractions from the assay mixture were identified by gel permeation chromatography in darkness when the methyl transfer from [^{14}C]methanol was terminated by the injection of 2 mM 1-iodopropane. The proteins showed apparent molecular masses of 40 to 60 kDa and 20 to 30 kDa (Fig. 2). The ^{14}C label was absent from the protein fractions, however, when the methyl transfer was stopped by rapidly chilling the incubation mixture in an ice bath.

Analyses of corrinoids from the ^{14}C-labeled enzyme fractions revealed a [^{14}C]cobamide that was more lipophilic than HO-pCC, which is the predominant corrinoid from whole cells. This finding suggested the formation of a [*methyl*-^{14}C]CH_3-pCC.

Two Co-methyl isomers might occur with pCC (Fig. 3), since this cobamide shows properties of an "incomplete" corrinoid. The in vitro methylation of pCC by methyl iodide showed approximately 30% Coα-CH_3-pCC and 70% Coβ-CH_3-pCC. Both isomers were separated by means of RP18-HPLC (Fig. 4A). Interestingly, the ^{14}C-labeled corrinoid from the [^{14}C]methanol incubation cochromatographed with the Coβ-methyl isomer (Fig. 4B). This radioactive corrinoid showed properties like those of the synthetic Coβ-CH_3-pCC. It was resistant to cyanolysis in 1 mM KCN at room temperature for 12 h in darkness, and it was converted into two CN-pCC isomers during a subsequent photolysis.

The methanol-activating system and the methyl transferases were investigated for their substrate specificities. Surprisingly, no detectable methylation of DL-tetrahydrofolate from CH_3-cobalamin was observed after a prolonged incubation for 3 h. In addition, ^{57}Co-labeled protein-bound

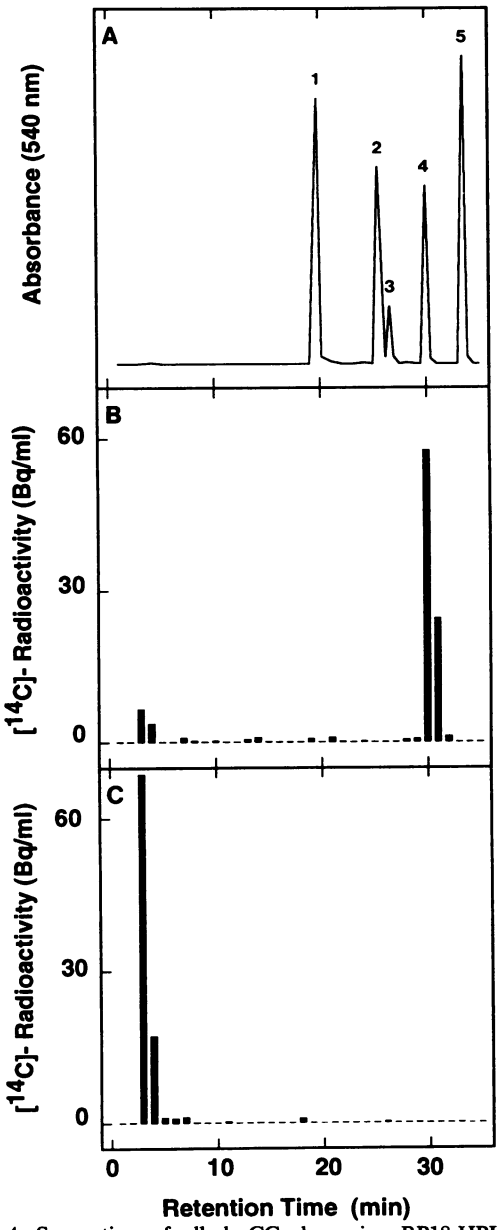

FIG. 4. Separation of alkyl-pCCs by using RP18-HPLC. (A) Authentic alkyl-pCCs. Peaks 1 and 2, CN-pCC isomers; peak 3, Coα-methyl-pCC; peak 4, Coβ-methyl-pCC; peak 5, Coβ-propyl cobamide. (B and C) Separation of ^{14}C-containing corrinoid extracts from proteins which catalyzed the methyl transfer from [^{14}C]methanol to 5-[^{14}C]methyl-tetrahydrofolate. (B) Corrinoid extract prepared in darkness. (C) Corrinoid extract after photolysis.

cresolyl cobamide cofactors were not exchanged during this incubation period for CH_3-cobalamin, HO-cobalamin, CH_3-pCC, or HO-pCC when these compounds were added to the assay mixture. Further, 3,4-dimethoxybenzoate was inactive as an in vitro methyl donor for the synthesis of 5-methyltetrahydrofolate or CH_3-cobalamin, although methoxyl groups serve as a carbon source for growing cells. These results indicate a specific methyl transfer pathway from methanol to DL-tetrahydrofolate.

Metabolism of methoxybenzoates. Five different meth-

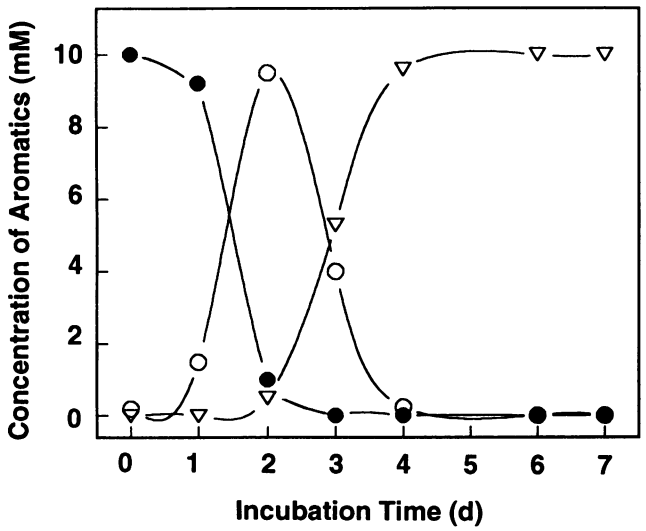

FIG. 5. Sequential O demethylation of 3,4-dimethoxybenzoate (●) via 4-hydroxy-3-methoxybenzoate (○) to 3,4-dihydroxybenzoate (▽) by growing *Sporomusa* cells. d, days.

oxyaromatics have been tested, and they were found to support growth of *S. ovata* (Table 2). The corresponding hydroxyaromatics were released into the medium because they were not further metabolized (see also Fig. 5). Growth yields and acetic acid formation strictly depended on the number of the available methoxyl groups. About 8.3 g of dry cell mass and 0.75 mol of acetic acid were formed per mol of methoxyl group in the presence of CO_2.

3,4-Dimethoxybenzoate metabolism was studied with growing *Sporomusa* cells and with proteins in vitro. Variable concentrations of two additional aromatic compounds appeared in the medium during growth with 3,4-dimethoxybenzoate (Fig. 5). Homogeneous samples of these compounds were identified as 4-hydroxy-3-methoxybenzoate and 3,4-dihydroxybenzoate on the basis of cochromatography of these aromatics with authentic references, UV-visible spectroscopy in solutions at pHs 3 and 11, and data from ^{1}H-NMR spectroscopic analyses. These methods clearly distinguished vanillate from isovanillate (see also Table 1), and thus the results are consistent with a sequential O demethylation of 3,4-dimethoxybenzoate via vanillate to gallic acid by growing *Sporomusa* cells.

Regulation of methyl metabolism. When methanol-adapted cells were grown in 120 mM methyl alcohol plus 10 mM 3,4-dimethoxybenzoate, they preferentially used methanol and demethylated only up to 0.3 mM methyl ether. The 12-fold-higher concentration of methanol compared with 3,4-dimethoxybenzoate did not prevent the *O*-methyl ether cleavage. This was proved by experiments with anaerobically washed cells that were resuspended in fresh medium supplemented with either 10 mM 3,4-dimethoxybenzoate or 120 mM methanol. Growth continued without any lag phase in methanol medium, but the O demethylation started after a delay of approximately 4 to 6 days. Dimethoxybenzoate-grown cells, however, immediately utilized methanol. This finding indicates that methanol metabolism in *S. ovata* is readily inducible, in contrast to methoxybenzoate utilization.

Methoxybenzoate utilization in vitro. Washed and air-oxidized proteins from 3,4-dimethoxybenzoate-grown cells strictly required DL-tetrahydrofolate as a methyl acceptor and a reductive activation by titanium citrate plus ATP in order to catalyze the O demethylation of 3,4-dimethoxybenzoate. The DL-tetrahydrofolate methylation occurred at a rate of 200 nmol min^{-1} mg of protein^{-1}, with apparent K_m values for the methyl donor 3,4-dimethoxybenzoate and the methyl acceptor DL-tetrahydrofolate of 450 and 330 μM, respectively. The transfer reaction could be inhibited by 2 mM 1-iodopropane. This 1-iodopropane inhibition and the reductive activation mechanism suggested a corrinoid-dependent methyl transfer, analogous to the methanol utilization. Indeed, two proteins with apparent molecular masses of 40 and 100 kDa became labeled in assays with 3,4-[4-*methoxyl*-^{14}C]dimethoxybenzoate when the reactions were terminated by 2 mM 1-iodopropane (Fig. 6). The radioactivity derived from Coβ-[*methyl*-^{14}C]CH_3-pCC cofactors of these proteins.

Soluble proteins from 3,4-dimethoxybenzoate-grown cells also demethylated 3,4,5-trimethoxybenzoate, 3-hydroxy-4-methoxybenzoate, syringate, ferulate, and 2-methoxybenzoate in the in vitro production of 5-methyl-tetrahydrofolate (Table 2). Significantly slower 5-methyl-tetrahydrofolate formation from methoxybenzene and from resorcin dimethyl ether, which has a substituent in the *meta* position, was

TABLE 2. Catabolism of methylated compounds by *S. ovata*

Methylated compound	Concn (mM)[a]	Reaction product[b]	Demethylation rate (nmol h^{-1} mg of protein^{-1})[c]
2-Methoxybenzoate	—	2-Hydroxybenzoate	192
3-Methoxybenzoate	—	ND	<1
4-Methoxybenzoate	—	ND	<1
3,4-Dimethoxybenzoate	10	3,4-Dihydroxybenzoate	240
3,4,5-Trimethoxybenzoate	10	3,4,5-Trihydroxybenzoate	264
4-Hydroxy-3,5-dimethoxybenzoate	6	3,4,5-Trihydroxybenzoate	216
4-Hydroxy-3-methoxycinnamate	5	3,4-Dihydroxycinnamate	204
1,2-Dimethoxybenzene	—	ND	372
1,3-Dimethoxybenzene	3	1,3-Dihydroxybenzene	12
Methoxybenzene	—	ND	7
CH_3-cobalamin	—	HO-cobalamin	<1

[a] Tested by growing cells. —, not tested by growing cells.
[b] ND, not determined.
[c] In vitro O-demethylation rate catalyzed by proteins from 3,4-dimethoxybenzoate-grown cells. The concentrations of the methoxyaromatics and of CH_3-cobalamin were 5 and 0.2 mM, respectively, in the assay mixture.

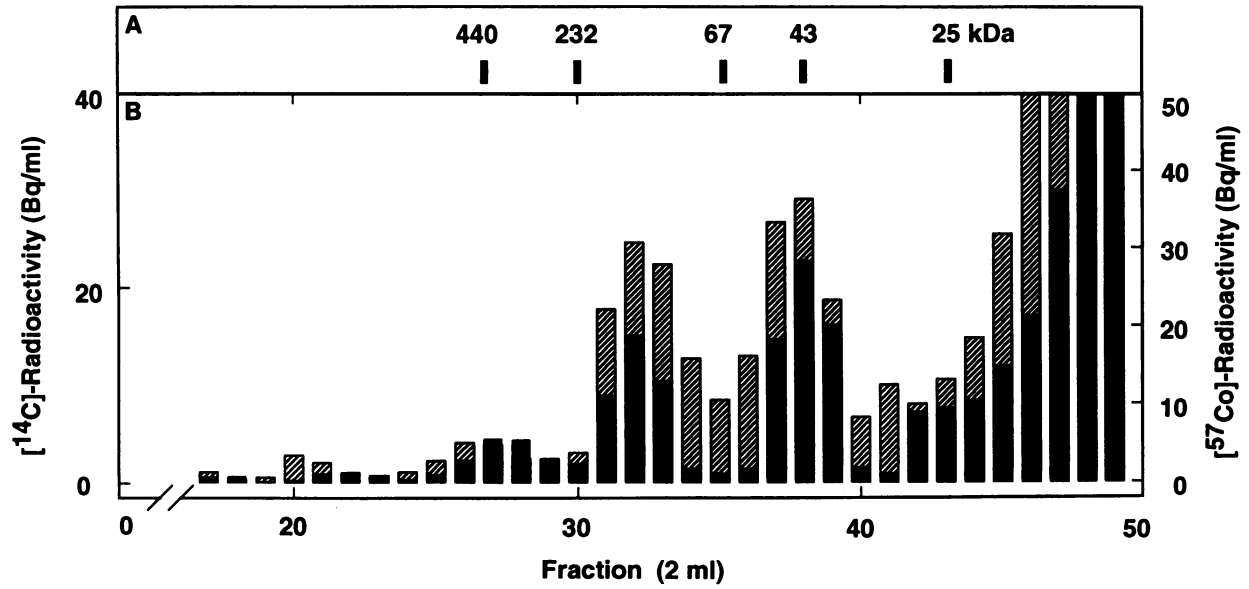

FIG. 6. Gel permeation chromatography of an in vitro assay for 5-methyl-tetrahydrofolate formation from 3,4-[4-*methoxyl*-^{14}C]dimethoxybenzoate. The reaction was catalyzed by ^{57}Co-labeled proteins which were not washed prior to the incubation. The methyl transfer was terminated by the addition of 1-iodopropane. (A) Molecular weight markers; (B) distributions of the ^{14}C radioactivity (■) and of the ^{57}Co radioactivity (▨).

observed. No detectable demethylation from 4-methoxybenzoate, 3-methoxybenzoate, α-D-3-*O*-methylglucose, or CH_3-cobalamin occurred within a 3-h incubation period (Table 2). Interestingly, 5 mM methoxybenzene did not prevent the O demethylation of 5 mM 3,4-dimethoxybenzoate.

Notably, 5-methyl-tetrahydrofolate was demethylated in the presence of cob(I)alamin, which was concomitantly converted into CH_3-cob(III)alamin. The reaction was catalyzed by enzymes from methanol-grown cells and by those from 3,4-dimethoxybenzoate-grown cells, at rates of 48 and 52 nmol min^{-1} mg of $protein^{-1}$, respectively. The K_m for 5-methyl-tetrahydrofolate was 630 μM at 30°C. This methyl transfer required a titanium citrate reduction but was independent of ATP.

DISCUSSION

Two anaerobic C-O bond cleavage reactions and the accompanying methyl transfers were investigated by using methanol and the *O*-methyl ether 3,4-dimethoxybenzoate. Both reactions proceeded via distinct corrinoid-dependent methyl transferases to the methyl acceptor DL-tetrahydrofolate. One of these homomeric methyl transferases appears to be identical to a previously characterized protein from methanol-grown cells (17). This corrinoid-containing enzyme was induced by methanol. It was immunologically evident in protein extracts from methanol-grown cells, but not in extracts from 3,4-dimethoxybenzoate-grown cells (17). Thus, a similar corrinoid-dependent methyl transferase in 3,4-dimethoxybenzoate-grown cells appears to be serologically different, although it catalyzes a homologous reaction (Fig. 7).

All *Sporomusa* proteins which are involved in anaerobic methanol or 3,4-dimethoxybenzoate metabolism seem to resist irreversible oxygen inactivation, because the methyl transfer activities to DL-tetrahydrofolate were restored after reduction. Titanium citrate is required at least for the reduction of oxidized corrinoid cofactors to the cobalt(I) corrinoids. Reduced cob(I)amides are the methyl acceptors, rather than the oxidized cob(II)amides and cob(III)amides, and redox potentials (E_0') as low as −500 mV might be expected for the protein-bound pCC, as determined by measurements with methyl transferases from *Clostridium thermoaceticum* (15) or the methionine synthetase from *Escherichia coli* (2).

The corrinoid-dependent methyl transferases in *S. ovata* did not supply the methyl groups directly onto added cob(I) alamin as a potential methyl trap, since cob(I)alamin could not substitute for DL-tetrahydrofolate as the methyl acceptor. In addition, 5-methyl-tetrahydrofolate formation vanished in the methanol and O-demethylation assays when these methyl donors were replaced by CH_3-cobalamin. These findings are consistent with a restricted methyl and cofactor exchange between the protein-bound CH_3-corrinoid

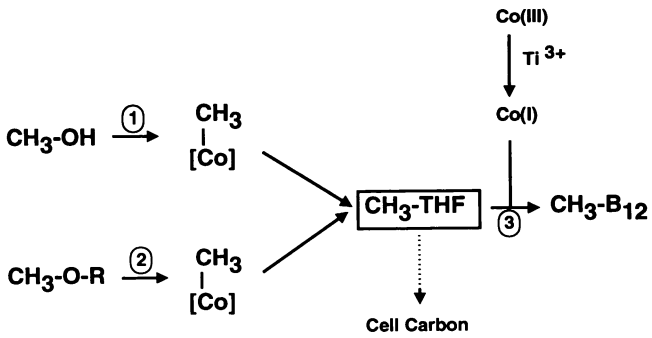

FIG. 7. Corrinoid-dependent methanol metabolism (reaction 1) and methoxybenzoate metabolism (reaction 2) by *S. ovata*. Each pathway might occur via more than one enzyme. Reaction 3 was used in the spectrophotometric quantification of the preceding methyl transfers in vitro. THF, tetrahydrofolate.

cofactors and various corrinoids which are added to the assay mixture. Furthermore, the intermediate concentration of the protein-bound Coβ-CH_3-pCC seems to be very low, indicating that the reaction promotes the demethylation of the corrinoid cofactor.

The methanol-activating proteins and the methoxybenzoate-demethylating enzymes showed a specific recognition mechanism for their substrates, in that methanol-grown cells were unable to use methoxybenzoates and 3,4-dimethoxybenzoate-grown cells did not activate methanol. A sequential O demethylation of 3,4-dimethoxybenzoate by growing cells suggested a specific activation mechanism even for various *O*-methyl ethers. Similar sequential O demethylations were reported for 3,4,5-trimethoxybenzoate metabolism by *Eubacterium limosum* (5) and for syringate metabolism by a carbon monoxide-activated enzyme system in *C. thermoaceticum* (22). However, a specific recognition mechanism was not apparent from these experiments.

Our in vitro experiments with *O*-methyl ethers provide evidence for a recognition mechanism based on vicinal substituents. Utilizable *O*-methyl donors in vitro provided either a vicinal methoxyl group, a hydroxy group, or a carboxyl group. Methoxyl substituents in aromatic *meta* positions were demethylated significantly more slowly by these enzymes, and 4-methoxybenzoate was not used as a substrate.

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