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# *O*-Demethylation by the homoacetogenic anaerobe *Holophaga foetida* studied by a new photometric methylation assay using electrochemically produced cob(I)alamin

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The previously studied complete methyl transfer sequence of tetrahydrofolate-dependent *O*-demethylation catalyzed by *Holophaga foetida* strain TMBS4 extracts was separated into two steps using cobalamins as non-physiological substrates: electrochemically produced cob(I)alamin served as methyl acceptor for phenyl methyl ether demethylation, yielding methylcob(III)alamin (reaction I), and methylcob(III)alamin served as donor for tetrahydrofolate methylation, yielding 5-methyl tetrahydrofolate (reaction II). Both reactions were measured with a new and direct photometric assay of cob(I)alamin methylation (or the reverse reaction) at 540 nm, the isosbestic wavelength of the cob(II)alamin/cob(I)alamin redox couple ($\Delta\varepsilon_{540} = 4.40\ mM^{-1} \cdot cm^{-1}$). The rates of reactions I and II were proportional to protein concentration, unlike the complete reaction sequence. Small components of cell extract did not affect activity of reactions I and II. Isovanillate demethylation by extracts of syringate-grown cells (reaction I) required reductive activation by cob(I)alamin and was inhibited and inactivated by cob(II)alamin, indicating that the reaction mechanism was a nucleophilic attack of an enzyme-bound corrinoid in the reduced Co(I) state on the methyl carbon of the ether, rather than a radical attack. Only phenyl methyl ethers were demethylated; demethylation rates were enhanced by *ortho*-hydroxyl or *para*-carboxyl groups, but reduced by additional *meta* substituents. The rate of isovanillate demethylation was 81 nmol · $min^{-1}$ · (mg protein)$^{-1}$ [0.76 mM cob(I)alamin] and apparent kinetic constants for cob(I)alamin were: $K_m = 1.2$ mM, $V_{max} = 220$ nmol · $min^{-1}$ · (mg protein)$^{-1}$, and $V_{max}/K_m = 180$ nmol · $min^{-1}$ · (mg protein)$^{-1}$ · $mM^{-1}$. 3,5-Dihydroxyanisole demethylation by extracts of 3,5-dihydroxyanisole-grown cells (also reaction I) was much slower. Reaction II did not require activation; specific activity and the specificity constant for methylcob(III)alamin were much lower.

Corrinoid-dependent methyl transfer reactions enable anaerobic bacteria to catabolize methylated substrates such as methyl ethers, methanol, methylated amines, and methylated sulfides. Corrinoid-dependent methyl transfers have been studied thoroughly with methanogenic bacteria. The methyl group of methanol is transferred to coenzyme M by the concerted action of two methyltransferases; the first one contains a corrinoid and must be reductively activated by an activation protein (Daas et al., 1993). $CH_3$-$H_4$methanopterin:coenzyme M methyltransferases are primary sodium pumps (Becher et al., 1992) that are integrated into membranes (Fischer et al., 1992). The study of methyl transfer to CO dehydrogenase in the homoacetogen *Clostridium thermoaceticum* (Lu et al., 1990) is much more advanced than our understanding of homoacetogenic demethylating enzyme systems (Berman and Frazer, 1992; Stupperich and Konle, 1993). We decided to investigate demethylation reactions with *Holophaga foetida* strain TMBS4 (Liesack et al., 1994) because of its interesting novel properties. (a) Methyl groups of methoxylated aromatic compounds have a branched metabolism (Bak et al., 1992; Kreft and Schink, 1993); the methyl groups can be either carbonylated to acetate involving CO dehydrogenase, or can be used to methylate sulfide to methanethiol ($CH_3SH$) and subsequently to dimethylsulfide ($CH_3$-S-$CH_3$). (b) The aromatic moiety liberated by demethylation is degraded to acetate through the phloroglucinol pathway (Kreft and Schink, 1993).

Our approach to the study of demethylation has been the following. We developed convenient and continuous assays for quantitative, kinetic studies in cell-free extracts that led to a working model of the demethylating enzyme system comprising the sequence of methyl donors and acceptors, the proteins involved, and their activation. We want to purify these proteins by using our assays and the information on both properties and activation in order to analyse the pure and active system. Finally, we want to prove that the pure system is complete by comparison with the complex cell extract system.

In this study, we report on a new and direct photometric assay for methyl transfer reactions of *O*-demethylation that is based on the use of cobalamins as non-physiological sub-

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*Abbreviations.* Buffer A, 50 mM potassium phosphate pH 7.2; SD, standard deviation; 5-$CH_3$-$H_4$folate, (6*RS*)-5-methyl-5,6,7,8-tetrahydrofolate; $H_4$folate, (6*RS*)-5,6,7,8-tetrahydrofolate.

strates. With cob(I)alamin as a methyl acceptor for demethylation of phenyl methyl ethers, and methylcob(III)alamin as a methyl donor for methylation of $H_4$folate, the previously studied complete *O*-demethylation reaction (Kreft and Schink, 1993; unpublished results) was split into two single methyl-transfer steps that were easier to study.

## MATERIALS AND METHODS

### Media and growth conditions

Strain TMBS4 (DSM 65 91) was grown at 28 °C in bicarbonate-buffered (pH 7.2), cysteine-reduced freshwater mineral medium free of sulfide, under a $N_2/CO_2$ atmosphere (80:20, by vol.) as previously described (Kreft and Schink, 1993). Media with 6 mM syringate or 3,5-dihydroxyanisole were inoculated and a few dithionite crystals were added (final concentration about 100 µM).

### Preparation and ultrafiltration of cell extracts

Cells were harvested anoxically during exponential growth using an anoxic chamber with $N_2/H_2$ atmosphere (95:5, by vol.). Washed cells were disrupted by French press treatment, the cell extract was centrifuged (15 min at 30000×*g*, 4 °C), and the supernatant was frozen in liquid $N_2$. The cell extract was ultrafiltered in a stirred cell with a YM 30 membrane (Amicon) inside an anoxic chamber at 20 °C.

### Methyl transfer assays

*Photometric assay for cob(I)alamin methylation*

Cob(I)alamin methylation was assayed in rubber-stoppered glass cuvettes under a $N_2$ atmosphere at room temperature (about 22 °C). Buffer A (50 mM potassium phosphate, pH 7.2) was freed of oxygen by bubbling with $N_2$. Oxygen was removed from other solutions by six cycles of evacuation and $N_2$-flushing. Dry crystals of oxygen-sensitive chemicals were filled into bottles, then gassed with $N_2$, and afterwards dissolved in anoxic buffer A. Anoxic reagents were added with gas-tight syringes (Unimetrics, Macherey & Nagel) which were rinsed with anoxic buffer A or reagent before use. Reduced cobalamins were transferred by syringes preincubated with the respective cobalamin solution in order to reduce the amount of oxygen that diffused out of the Teflon plugs. The standard reaction mixture contained the following components (final concentrations), which were added in this sequence: buffer A, 40 mM isovanillate, extract of syringate-grown cells (0.6 mg protein · $ml^{-1}$), and about 0.75 mM cob(I)alamin. The exact concentration of cobalamin stock solutions in the electrochemical cells had to be determined after each experiment. Reagent stock solutions were prepared with buffer A and methoxylated acids were neutralized with NaOH. Cuvettes with only a 5-mm light path were used (400 µl liquid in 770 µl total volume) to accommodate high cobalamin concentrations.

*Photometric assay for $CH_3$-cobalamin demethylation*

Assays were performed as described above, but the composition of the assay mixture was altered. The standard reaction mixture contained the following components (final concentrations), which were added in this sequence: buffer A, 0.5 mM $CH_3$-cobalamin, extract of syringate-grown cells (2.2 mg protein · $ml^{-1}$), and 10 mM $H_4$folate. Reagent stock solutions of $H_4$folate were prepared with buffer A in amber serum bottles and neutralized with five equivalents of KOH. Bottles containing $CH_3$-cobalamin were wrapped with aluminium foil.

*Discontinuous methyl transfer assays*

Assays were performed as described above but with other concentrations of methyl donors (1 mM) and acceptors (1 mM). Samples were removed from reaction mixtures with syringes and analysed by HPLC.

*Calculation of kinetic parameters*

Michaelis-Menten or other equations were fitted to untransformed data by a least-squares algorithm.

### Electrochemical reduction of cob(III)alamin

Aquocob(III)alamin dissolved in buffer A was reduced in a three-compartment electrochemical cell with a gold foil working electrode (area 2 $cm^2$, thickness 0.1 mm, purity 99.998%) and Ag/AgCl (3 M KCl) reference and counter electrodes. Reference and counter electrode compartments were connected to the working electrode compartment (containing about 10 ml magnetically stirred liquid) by agar salt bridges (3% agar in 3 M KCl) that were mechanically supported by glass frits. The cell was a glass construction sealed with butyl rubber stoppers. Electrode wires were passed through the stoppers and connected to a laboratory potentiostat (Wenking model LB 81 M, Bank Elektronik). The parts were assembled in an anoxic chamber and the headspace was flushed with nitrogen afterwards. The reference electrode was made by anodizing a silver wire in 0.1 M HCl according to the first protocol given in Sawyer and Roberts (1974). The cell potential was poised to −810 mV for the reduction of aquocob(III)alamin (8 mM) to cob(I)alamin ($E_0'$ = −610 mV; Dryhurst et al., 1982). To produce cob(II)alamin ($E_0'$ = 202 mV; Dryhurst et al., 1982), we first reduced aquocob(III)alamin (2 mM) to cob(I)alamin, and subsequently poised the solution to −205 mV (i.e. the midpoint between above-mentioned standard potentials). All potentials are expressed versus the normal hydrogen electrode.

### Analytical methods

*Photometry*

Spectra were recorded at room temperature in buffer A under an $N_2$ atmosphere [except for cob(III)alamin] in 3-ml quartz cuvettes with a Uvikon 930 (Kontron) photometer set to a 2-nm spectral bandwidth; baselines of buffer-filled cuvettes were recorded before the cobalamin stock solutions were added. Cuvettes were closed with a taper joint glass construction with a side-arm, ending in a two-way stopcock for connection to a $N_2$ line, and a taper joint glass plug, allowing additions to be made under a positive $N_2$ pressure. The isosbestic wavelength was determined in rubber-stoppered cuvettes. After a baseline run, cob(I)alamin was added to buffer A and the absorbance was recorded with a fixed-wavelength programme from 535 nm to 545 nm in 1-nm intervals. Then, 5 µl air was injected and the absorbance was measured repeatedly until only a small fraction of cob(I)-

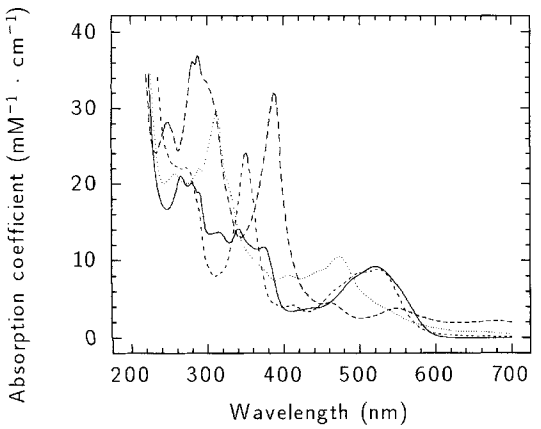


**Fig. 1. Spectra of cobalamins in 50 mM potassium phosphate pH 7.2.** Methylcob(III)alamin (———), aquocob(III)alamin (----), cob(II)alamin (· · · · ·), and cob(I)alamin (– – – –).

alamin was left. Fast spectral scans confirmed that only cob(I)alamin and cob(II)alamin were present. To measure absorption coefficients, absorbances were baseline-corrected at 700 nm to avoid the possible contribution of dirt to absorbance values. Therefore, cob(I)alamin was converted to dicyanocobalamin before this baseline correction. $CH_3$-cobalamin solutions were always prepared anoxically in a dark room before measurements. The concentration of each cobalamin sample was determined afterwards.

*Corrinoid determination*

Cyanocob(III)alamin, aquocob(III)alamin, and $CH_3$-cobalamin were converted to dicyanocob(III)alamin that was quantified photometrically (modified from Fischer et al., 1992); KCN (100 µl 1 M) was added to 1 ml of sample or standard in a small plastic tube. These solutions were illuminated for 10 min (the projector lens of a cold light lamp was placed above the open tubes) in order to split off the methyl groups from $CH_3$-cobalamin. Then, samples were filled into cryo screw tubes that could be sealed tightly and boiled in a water bath for 15 min. After cooling, samples were poured into polystyrene cuvettes and incubated for 2 h in the dark, then absorbance differences ($\Delta A_{580-640}$) were measured. Since absorbances of all samples drifted slightly at the same rate, the measurement time table was planned in such a way that time differences were almost cancelled. Absorbance yields of cyanocob(III)alamin and aquocob(III)alamin standards were the same.

*HPLC*

Aromatic compounds, cobalamins, and folates were analysed by $C_{18}$ reverse-phase HPLC and photometric detection as previously described (Brune and Schink, 1990). Folate specimens were handled anoxically; $CH_3$-cobalamin samples were protected from light.

*Protein assay*

Protein concentrations were determined by the improved Bradford procedure of Read and Northcote (1981) with dye-reagent no. 1 and with ovalbumin as standard.

**Table 1. Activity of methyl transfer from isovanillate to cob(I)alamin or from $CH_3$-cobalamin to $H_4$folate in untreated, ultrafiltered, and reconstituted cell extract of *Holophaga foetida*.** Due to the ultrafiltration procedure, the filtrate contained small molecules of the cell extract in 10-fold dilution, and the retentate was depleted of small molecules by about 100-fold. We used 5 µl extract of syringate-grown cells with 42.6 ± 2.5 mg protein · $ml^{-1}$, 5 µl retentate with 40.7 ± 1.4 mg protein · $ml^{-1}$, and 200 µl filtrate with 0.03 ± 0.01 mg protein · $ml^{-1}$ in an assay volume of 400 µl, except for $CH_3$-cobalamin demethylation for which we used 20 µl cell extract and retentate. In-experiment variation ($n$ = 2) is given as SD.

| Fraction | Specific activity | |
|---|---|---|
| | isovanillate → cob(I)alamin | $CH_3$-cobalamin → $H_4$folate |
| | nmol · $min^{-1}$ · (mg protein)$^{-1}$ | |
| Cell extract | 59 ± 1.1 | 0.79 ± 0.01 |
| Filtrate | 2 ± 0.7 | 0.00 ± 0.00 |
| Retentate | 57 ± 1.3 | 0.89 ± 0.01 |
| Retentate + filtrate | 60 ± 0.7 | 0.89 ± 0.00 |

## Materials

Oxygen traces were removed from nitrogen by passing the gas over a BASF R-3-11 catalyst train (Broadbent, 1967). Titanium(III) nitrilotriacetate stock solutions were prepared in an anoxic chamber after Moench and Zeikus (1983). The solution (pH 7.2) contained 100 mM $Ti^{3+}$ chelated by 150 mM nitrilotriacetate, as well as about 600 mM KCl. (6*RS*)-5,6,7,8-Tetrahydrofolate · 3 HCl and (6*RS*)-5-methyl-5,6,7,8-tetrahydrofolate (Ca salt) were purchased from Merck.

## RESULTS

We developed a direct photometric assay for methyl transfer reactions involved in *O*-demethylation and determined the prerequisites of the assay. Then we applied this assay (a) to the study of cob(I)alamin-dependent phenyl methyl ether demethylation with extracts of syringate- or 3,5-dihydroxyanisole-grown cells, and (b) to the study of $H_4$-folate-dependent $CH_3$-cobalamin demethylation with extracts of syringate-grown cells.

### Photometric methyl transfer assays

The principle of the photometric assay for cob(I)alamin methylation or the reverse reaction can be deduced from the absorption spectra of the cobalamins (Fig. 1); methylation of cob(I)alamin to methylcob(III)alamin increases the absorbance at the long-wavelength maximum of $CH_3$-cobalamin. Oxidation of cob(I)alamin by components of the reaction mixture and by oxygen leaking into the vessel results in the presence of cob(II)alamin, even if pure cob(I)alamin was supplied. A shift of the cob(II)alamin/cob(I)alamin redox equilibrium, caused by methylation of cob(I)alamin, cannot interfere with the cob(I)alamin methylation measurement at the isosbestic wavelength of the cob(II)alamin/cob(I)alamin redox couple. Cob(III)alamin formation would interfere with measurements and must therefore be avoided.

We determined this isosbestic wavelength in potassium phosphate pH 7.2 at room temperature, avoiding errors

**Table 2. Activity of extracts of syringate–grown cells for cob(I)alamin-dependent demethylation of various methyl donors.** The specific activity of isovanillate demethylation, i.e. 100%, was $62 \pm 1.5$ nmol · min$^{-1}$ · (mg protein)$^{-1}$ ($\bar{x} \pm$ SD). The non-aromatic compounds methoxyethanol, methoxyacetate, methanol, betaine, and trimethylamine were not significantly demethylated, i.e. activity was in the range of the control values $-0.05 \pm 0.25$ % ($\bar{x} \pm 3 \times$SD). Note that 3,4,5-trimethoxybenzoate can be demethylated at several non-equivalent sites; hence, the relative substitution pattern is equivocal and the observed rate is an average for the three methoxyl groups. However, although syringate carries two methoxyl groups, the measured rate can be viewed as one per methoxyl group since both are equivalent. In-experiment variation ($n = 2$) is given as SD.

| Methyl donor (10 mM) | Substitution pattern relative to the site of demethylation | | | Relative activity |
|---|---|---|---|---|
| | *ortho* | *meta* | *para* | |
| Isovanillate | OH,– | –,– | $COO^-$ | 100 ±2.9 |
| Vanillate | OH,– | $COO^-$,– | – | 35 ±1.7 |
| Guaiacol | OH,– | –,– | – | 56 ±1.6 |
| 3,4,5-Trimethoxybenzoate | $OCH_3$,$OCH_3$ | –,– | $COO^-$ | 88 ±1.8 |
| Syringate | OH,– | $OCH_3$,$COO^-$ | – | 41 ±0.39 |
| 5-Hydroxyvanillate | OH,– | OH,$COO^-$ | – | 35 ±0.64 |
| 3-Methoxycatechol | $OH^-$,– | OH,– | – | 9.1 ±0.18 |
| 2-Methoxybenzoate | $COO^-$,– | –,– | – | 1.4 ±0.13 |
| 3,5-Dihydroxyanisole | –,– | OH,OH | – | 1.2 ±0.02 |
| 3-Hydroxyanisole | –,– | OH,– | – | 1.9 ±0.04 |
| 3-Methoxybenzoate | –,– | $COO^-$,– | – | 2.2 ±0.32 |
| 4-Hydroxyanisole | –,– | –,– | OH | 1.1 ±0.02 |
| 4-Methoxybenzoate | –,– | –,– | $COO^-$ | 6.2 ±0.13 |
| 4-Methoxyphenylacetate | –,– | –,– | $CH_2COO^-$ | 0.5 ±0.06 |
| 4-Methoxyphenylpropionate | –,– | –,– | $CH_2CH_2COO^-$ | 0.5 ±0.07 |
| Anisole | –,– | –,– | – | 1.9 ±0.17 |

owing to concentration measurements, cob(III)alamin formation, baseline differences, dirt introduced with the cobalamin sample, and distortions of the spectrum shape due to scanning. Isosbestic wavelengths were (mean ± SD, $n = 2$): 539.85 ±0.15 nm (measured with a Kontron Uvikon 930 photometer), and 540.30 ± 0.15 nm (measured with a Kontron Uvikon 860 photometer). We used a rounded value of 540 nm for our experiments.

Absorption coefficients in potassium phosphate pH 7.2 at room temperature and 540 nm were (mean ± SEM): 3.29 ±0.02 mM$^{-1}$ · cm$^{-1}$ for cob(I)alamin, 7.70 ± 0.02 mM$^{-1}$ · cm$^{-1}$ for $CH_3$-cobalamin, and 4.40 ± 0.03 mM$^{-1}$ · cm$^{-1}$ for cob(I)alamin methylation (i.e. $\Delta\varepsilon_{540}$).

$CH_3$-cobalamin was not light-sensitive under assay conditions (monochromatic light of long wavelength, anoxic solution); the absorbance of a complete $CH_3$-cobalamin-containing reaction mixture without cell extract or methyl acceptor did not change within an hour. Spectra recorded at the beginning and after 1 h of incubation of $CH_3$-cobalamin (in buffer A with or without $Ti^{3+}$) in the light path of the photometer overlapped completely.

## Cob(I)alamin-dependent *O*-demethylation

### *Extract of syringate-grown cells*

Methyl transfer from phenyl methyl ethers, e.g. isovanillate (3-hydroxy-4-methoxybenzoate), to cob(I)alamin (reaction I) occurred only in the presence of cell extract and both substrates. Cob(I)-alamin was methylated to $CH_3$-cobalamin, which was identified by HPLC on the basis of co-elution and on-line ultraviolet/visible spectra. Cell extracts incubated for 5 min at 80 °C were inactive. Addition of 1 mM $H_4$folate to the reaction mixture left activity unchanged. Ultrafiltration and reconstitution of the cell extract demonstrated that small components of the cell extract did not affect isovanillate demethylation activity (Table 1). Various phenyl methyl ethers served as methyl donors for cob(I)alamin methylation; these activities were enhanced by *ortho*-hydroxyl or *para*-carboxyl groups, but reduced by additional *meta* substituents (Table 2). Non-aromatic methoxylated compounds were not demethylated. If the activity was low, e.g. with poor methyl donors or at low cob(I)alamin concentrations, an initial phase of increasing activity was observed (lag phase).

The activity of cob(I)alamin-dependent isovanillate demethylation increased essentially proportionally with protein concentration (Fig. 2A), in contrast to the previously studied $H_4$folate- or $CH_3SH$-dependent demethylation reactions (Kreft and Schink, 1993; unpublished results). The slight levelling-off of activity could be due to oxidation of the methyl acceptor cob(I)alamin by cell extract, and was considered by fitting hyperbolae to the data. At a cob(I)-alamin concentration of 0.76 mM, the specific activity (i.e. the initial slope of the dashed hyperbola in Fig. 2A) was 81 nmol · min$^{-1}$ · (mg protein)$^{-1}$. Activity was not markedly affected by including ATP (10 mM), $Mg^{2+}$ (1 mM), and $Ti^{3+}$ (12.5 mM) in the assay mixture (Fig. 2A).

We studied the dependence of the isovanillate demethylating activity on cob(I)alamin (Fig. 2B). The volume activity at a particular protein concentration fitted a Michaelis-Menten equation only if this equation was extended by a parameter allowing an intercept on the ordinate apart from the origin (Fig. 2B). Hence, for each of the ten cob(I)alamin concentrations, the volume activities obtained at three different protein concentrations were extrapolated to zero protein concentration (cf. Fig. 2A for an example of the procedure). These extrapolated (specific) activities were plotted versus cob(I)alamin concentration (Fig. 2B). The curve shows that

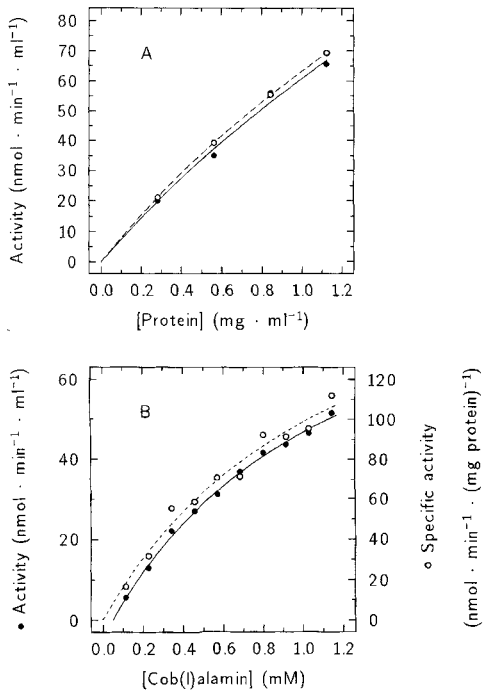


**Fig. 2. Protein and cob(I)alamin dependence of rates of methyl transfer from isovanillate to cob(I)alamin.** (A) Protein dependence. Activity without (○---○) or with (●——●) ATP (10 mM), $Mg^{2+}$ (1 mM), and $Ti^{3+}$ (12.5 mM) in the reaction mixture. The curves are hyperbolae fitted to the data. (B) Cob(I)alamin dependence. (●——●) Increase of volume activity with cob(I)alamin concentration at a protein concentration of 0.56 mg · $ml^{-1}$. The curve is a fit of a Michaelis-Menten equation, extended by a parameter allowing the equation to intercept the ordinate away from the origin, to the data. (○---○) Increase of specific activity with cob(I)alamin concentration. Activities at different protein concentrations [each at the same cob(I)alamin concentration] were extrapolated to zero protein concentration. The curve is a fit of an ordinary Michaelis-Menten equation to these extrapolated data.

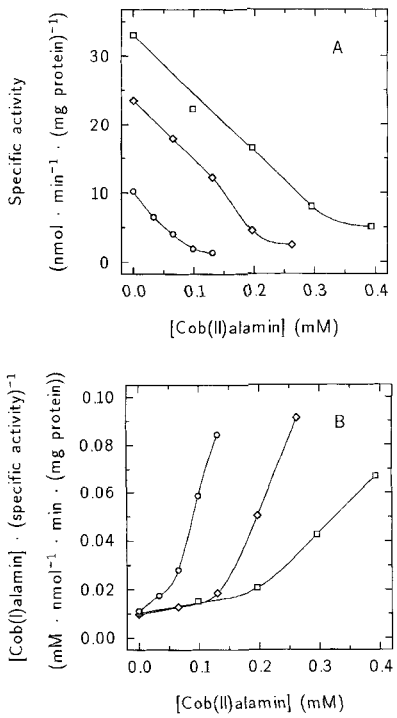


**Fig. 3. Inhibition of cob(I)alamin-dependent isovanillate demethylation by cob(II)alamin.** The fixed concentrations of the substrate cob(I)alamin were: 0.11 mM (○), 0.23 mM (◇), and 0.34 mM (□). (A) Plot of untransformed data; (B) plot after Cornish-Bowden (1976).

an ordinary Michaelis-Menten equation, which crosses the origin, fitted the data. The kinetic parameters of this fit were: apparent $K_m$ = 1.2 mM, apparent $V_{max}$ = 220 nmol · $min^{-1}$ · (mg protein)$^{-1}$, and apparent $V_{max}/K_m$ = 180 nmol · $min^{-1}$ · (mg protein)$^{-1}$ · $mM^{-1}$.

Cob(II)alamin inhibited the cob(I)alamin-dependent demethylation of isovanillate (Fig. 3A). Inhibition was neither competitive, mixed, nor uncompetitive, i.e. not of any type of linear inhibition that gives a set of straight lines (each line at fixed substrate concentration) in plots of (a) substrate concentration divided by velocity against inhibitor concentration (Fig. 3B) and (b) reciprocal velocity against inhibitor concentration (not shown; Cornish-Bowden, 1976).

*Extract of 3,5-dihydroxyanisole-grown cells*

We used 10 mM 3,5-dihydroxyanisole for these assays with cob(I)alamin as methyl acceptor. The rate of 3,5-dihydroxyanisole demethylation was not altered by $H_4$folate (1 mM) or $Ti^{3+}$ (1.25 mM). Reaction progress curves displayed a lag phase, i.e. activity increased at the beginning to reach the maximal value later. Increase of velocity with protein concentration was linear, and the specific activity was only 1.6 nmol · $min^{-1}$ · (mg protein)$^{-1}$.

### $H_4$folate-dependent $CH_3$-cobalamin demethylation

Methyl transfer from $CH_3$-cobalamin to $H_4$folate (reaction II) was investigated with extracts of syringate-grown cells and required cell extract as well as both substrates for activity. $H_4$folate was methylated to 5-$CH_3$-$H_4$folate, which was identified by HPLC on the basis of co-elution and online ultraviolet/visible spectra. Cell extracts incubated for 5 min at 80°C were inactive. Small components of cell extract did not alter $CH_3$-cobalamin demethylation activity as shown by ultrafiltration and reconstitution of the cell extract (Table 1). Activity was maximal from the start, i.e. a lag phase was not observed. The reaction rate increased proportionally with protein concentration; the specific activity was only 0.89 nmol · $min^{-1}$ · (mg protein)$^{-1}$. Including ATP (10 mM), $Mg^{2+}$ (1 mM), and $Ti^{3+}$ (12.5 mM) in the assay mixture did not change demethylation rates.

Extracts of syringate-grown cells also catalyzed methyl-cob(III)alamin formation from 5-$CH_3$-$H_4$folate and cob(I)-alamin. This reaction, however, was dependent on addition of the $Ti^{3+}$ reagent and, hence, cannot be the reverse of reaction II. This activity has not yet been further characterized.

The apparent specificity constant (apparent $V_{max}/K_m$) of the $CH_3$-cobalamin demethylating activity for its substrate $CH_3$-cobalamin was so low that we could determine neither apparent $K_m$ nor apparent $V_{max}$: the reaction rate increased proportionally with $CH_3$-cobalamin concentration from zero to 0.5 mM with a slope (apparent $V_{max}/K_m$) of 1.8 nmol · $min^{-1}$ · (mg protein)$^{-1}$ · $mM^{-1}$.

## DISCUSSION

We have developed a photometric assay to examine, e.g. substrate specificity, substrate dependence, protein depen-

dence, and redox dependence of the two methyl transfer steps that constitute the complete $H_4$folate-dependent *O*-demethylation reaction.

The direct photometric assay for cob(I)alamin methylation or $CH_3$-cobalamin demethylation required (a) strict exclusion of oxygen to avoid cob(III)alamin formation; (b) recording absorbance changes at the isosbestic wavelength (540 nm) of the cob(II)alamin/cob(I)alamin redox couple; (c) determination of the difference absorption coefficient ($\varepsilon_{\text{methylcob(III)alamin}} - \varepsilon_{\text{cob(I)alamin}}$) at this wavelength; and (d) insensitivity of $CH_3$-cobalamin against illumination at 540 nm under anoxic conditions. This assay could be applied as well to other corrinoid-dependent methyl transfer reactions. $Ti^{3+}$ is not required if the methyl-accepting cobalamin is reduced electrochemically. $Ti^{3+}$ should be avoided with phenyl methyl ethers which chelate $Ti^{3+}$ or give rise to $Ti^{3+}$ chelators upon demethylation (Kreft and Schink, 1993); these $Ti^{3+}$ complexes render the reaction mixture yellow (interfering with photometric measurements even at 540 nm; data not shown), and substrate concentrations would be reduced to unknown extents.

The complete methyl-transfer sequence from phenyl methyl ether to $H_4$folate is catalyzed by two (or more) proteins in *H. foetida* extracts (Kreft and Schink, 1993; unpublished results); the reaction rate is not affected by ultrafiltrate of the extract (unpublished results). This complete reaction requires reductive activation by $Ti^{3+}$ that is stimulated by ATP, $Mg^{2+}$ and protein(s) of the cell extract; propyl iodide inhibition indicated corrinoid dependence of *O*-demethylation (Kreft and Schink, 1993). Here, we studied the methyltransfer sequence from phenyl methyl ethers, e.g. isovanillate, to cob(I)alamin (reaction I) and from $CH_3$-cobalamin to $H_4$folate (reaction II). The rate of both reactions increased proportionally with protein concentration and small components of the cell extract had no effect on activity, implying that the reactions were each catalyzed by single enzymes. Preliminary results with chromatography fractions enriched with reaction-I-catalyzing activity showed that these fractions catalyzed neither reaction II nor the complete reaction (data not shown). After disruption of cells by French press treatment and subsequent ultracentrifugation, the reaction-I-catalyzing activity was found only in the supernatant (data not shown). Reaction I required reductive activation by cob(I)alamin, as inferred from the lag phase of the reaction, in contrast to reaction II. Propyl iodide inhibition, reductive activation by $Ti^{3+}$ or cob(I)alamin, and inactivation by cob(II)alamin (see below), support the view that reaction I is corrinoid-dependent. *H. foetida* contains high amounts of the corrinoid 5-hydroxybenzimidazolylcobamide (Factor III; Kreft and Schink, 1993). We conclude that reactions I and II constitute the complete reaction chain (Fig. 4).

Cob(II)alamin inhibited cob(I)alamin-dependent demethylation of isovanillate in a non-linear manner and not in a competitive manner as might have been expected for a compound with a substrate-resembling structure. This suggests that cob(II)alamin acted on several states of enzyme I (Fig. 4), e.g. inactivating the reduced and active enzyme by oxidation, inhibiting activation, and inhibiting cob(I)alamin binding to the methylated and active enzyme. Cob(I)alamin, but not cob(II)alamin, was able to accept methyl groups and activate the enzyme by reduction. This implies that the reaction mechanism is a nucleophilic attack of an enzyme-bound corrinoid in the reduced Co(I) state on the methyl carbon of the ether (the methyl group is cleaved off as a cation), rather than a radical attack. Cob(II)alamin inhibition led to experimental problems since oxidation of cob(I)alamin to cob(II)alamin was caused primarily by cell extract. This explains the need for extrapolation of activities measured at several protein concentrations to zero protein concentration (Fig. 2).

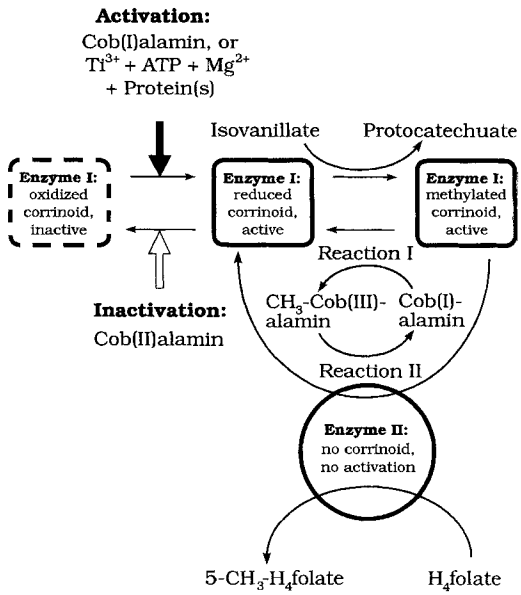


**Fig. 4. Working model of the demethylating enzyme system of *Holophaga foetida*.** This model summarizes conclusions of the present and of previous studies. The corrinoid-containing enzyme I undergoes an activation/inactivation and a methylation/demethylation cycle. It can be measured separately from the complete reaction, which is catalyzed by both enzymes, using cob(I)alamin as an artificial substrate [reaction I: isovanillate + cob(I)alamin → protocatechuate + methylcob(III)alamin]. Enzyme II, which does not contain a corrinoid, requires no activation and can be measured separately with methylcob(III)alamin as an artificial substrate [reaction II: methylcob(III)alamin + $H_4$folate → cob(I)alamin + 5-$CH_3$-$H_4$folate]. Both enzymes co-operate to catalyze demethylation of phenyl methyl ethers such as isovanillate with $H_4$folate as acceptor (complete reaction: isovanillate + $H_4$folate → protocatechuate + 5-$CH_3$-$H_4$folate).

Although the basic properties of isovanillate demethylation by extracts of syringate-grown cells and 3,5-dihydroxyanisole demethylation by extracts of 3,5-dihydroxyanisole-grown cells were similar (both are examples of reaction I), the rate of isovanillate demethylation was 50-fold higher and, hence, studied preferentially; the $H_4$folate-dependent $CH_3$-cobalamin demethylation (reaction II), however, was even slower. A lower activity of reaction I coincided with a lower activation rate, i.e. with a longer lag phase. The apparent specificity constant ($V_{max}/K_m$) of the isovanillate-demethylating activity for cob(I)alamin was 100-fold higher than the specificity of the $CH_3$-cobalamin-demethylating activity for $CH_3$-cobalamin; this might explain the observed differences in specific activities.

The specificity of the demethylating enzyme system (entire reaction) for phenyl methyl ethers was different in extracts of 3,4,5-trimethoxybenzoate- and 3,5-dihydroxyanisole-grown cells (unpublished results). Since the rates of these entire reactions are controlled by substrate concentration and also by protein concentration, this work had to remain qualitative. Here, we could examine the more simple system of reaction I quantitatively, but only with the sufficiently active extract of syringate-grown cells. As far as

studied, no qualitative differences between specificities of extracts of 3,4,5-trimethoxybenzoate- and syringate-grown cells were found (unpublished results; this study).

In conclusion, we have developed a direct photometric assay for corrinoid-dependent methyl-transfer reactions. The use of cobalamins as substrates allowed us to separate the entire methyl transfer sequence of *O*-demethylation into two parts catalyzed by single enzymes (Fig. 4) that were easier to study because of (a) the protein concentration independence of specific activity and of the enzyme I-activation rate, and (b) the protein concentration independence of specific activity and the lack of activation requirements of enzyme II. Inhibition and inactivation of *O*-demethylation by cob(II)-alamin proved that an active form of enzyme I contained a corrinoid in the nucleophilic Co(I) redox state.

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