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Specificity of *O*-demethylation in extracts of the homoacetogenic *Holophaga foetida* and demethylation kinetics measured by a coupled photometric assay

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Abstract The kinetics and specificity of *O*-demethylation were studied in cell-free extracts of the strictly anaerobic, methanethiol- and dimethylsulfide-producing homoacetogen *Holophaga foetida* strain TMBS4 with methanethiol and tetrahydrofolate (H_4folate) as methyl acceptors. Extracts of cells grown with 3,4,5-trimethoxybenzoate contained an enzyme system that demethylated various phenyl methyl ethers with at least one *ortho*-positioned hydroxyl or methoxyl group (the *ortho* system) and also contained a decarboxylase. Extracts of cells grown with 3,5-dihydroxyanisole contained an enzyme system with a novel specificity that demethylated only the *meta*-hydroxylated compounds 3,5-dihydroxyanisole and 3-hydroxyanisole (the *meta* system) and lacked a decarboxylase. H_4folate-dependent demethylation produced CH_3-H_4folate. For a photometric in vitro assay of the *meta* system, the NADPH-consuming phloroglucinol reductase (PR) reaction was coupled to the phloroglucinol-yielding demethylation of 3,5-dihydroxyanisole. The kinetics of the indicator enzyme PR were studied. The cell extract had a high and stable specific PR activity. PR was inhibited by phloroglucinol (substrate inhibition) and the substrate analogue 3,5-dihydroxyanisole. Doubling the PR activity of the coupled enzyme assay by additions of a PR-enriched fraction had no effect, showing that the PR activity supplied by cell extract did not limit reaction rates. Demethylation activity of the *meta* system with either methyl acceptor increased with the square of the protein concentration. With H_4folate, the in vivo activity could be attained. Kinetic parameters for the methyl acceptors were determined.

Dedicated to Prof. Achim Kröger on the occasion of his 60th birthday

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Abbreviations *PR* Phloroglucinol reductase · *H_4folate* Tetrahydrofolate

Introduction

Anaerobic bacteria demethylate phenyl methyl ethers by *O*-demethylation (DeWeerd et al. 1988). In *Acetobacterium woodii* cell extracts, ATP and tetrahydrafolate (H_4folate) are necessary for the air-sensitive demethylation reaction (Berman and Frazer 1992). In *Sporomusa ovata* cell extracts, demethylation is corrinoid-dependent, needs reductive activation by Ti^{3+} and ATP, and yields CH_3-H_4folate (Stupperich and Konle 1993). In strain MC cell extracts, demethylation yields CH_3-H_4folate and requires ATP substoichiometrically (Meßmer et al. 1993, 1996). CH_3-H_4folate production can be measured by a coupled enzyme assay (Meßmer et al. 1996). In *Clostridium thermoaceticum* cell extracts, syringate is demethylated by an oxygen-insensitive, corrinoid-independent, ATP-consuming enzyme yielding CH_3-H_4folate (El Kasmi et al. 1994).

We investigated demethylation reactions with *Holophaga foetida* strain TMBS4 (Bak et al. 1992). *H. foetida* combines the metabolic capabilities of demethylating homoacetogens and of trihydroxybenzene-degrading fermenting bacteria, but it is not phylogenetically related to such species (Liesack et al. 1994); it also methylates inorganic sulfide to methanethiol and, subsequently, to dimethylsulfide (Bak et al. 1992). In *H. foetida* cell extracts, demethylation needs reductive activation by Ti^{3+} and is additionally stimulated by ATP and cell extract, is corrinoid-dependent, can employ sulfide or methanethiol instead of H_4folate as the methyl acceptor, and its specific activity increases with protein concentration (Kreft and Schink 1993, 1994).

Here we report on a novel demethylating enzyme system with *meta* specificity. The development and results of a photometric assay for the *meta* system are described.

Materials and methods

Growth conditions and preparation of cell extracts

Holophaga foetida strain TMBS4 (DSM 6591) was grown, and cells were harvested anoxically during exponential growth using an anoxic chamber as described previously (Kreft and Schink 1994).

Enrichment of phloroglucinol reductase

Phloroglucinol reductase was enriched under cooling and air using a procedure modified from Haddock and Ferry (1989). Cells were grown with 5 mM phloroglucinol, harvested, and washed in 50 mM potassium phosphate (pH 7.2); the cell paste (1.5 g) was stored in liquid N_2, thawed, and resuspended in 50 mM Tris-HCl (pH 7.2) with 5% (w/v) glycerol; cells were disrupted by French press treatment as described (Kreft and Schink 1994). The cell extract was ultracentrifuged (15 min at 300,000 × *g*), and the supernatant was applied to a 6-ml Resource Q anion exchange column (Pharmacia, Freiburg, Germany). The enzyme was eluted with a linear KCl gradient (0–1 M in 20 bed volumes) in 50 mM Tris-HCl (pH 7.2) with 5% (w/v) glycerol at 30 mM KCl. The elution buffer was exchanged with start buffer by ultrafiltration (YM 10 membrane; Amicon, Witten, Germany). Aliquots of the retentate were stored in liquid N_2 and thawed and gassed with N_2 before use.

Enzyme assays

Photometric assay for 3,5-dihydroxyanisole demethylation

The 3,5-dihydroxyanisole-demethylating enzyme system of cell extracts was measured by coupling the formation of the product phloroglucinol to the phloroglucinol- and NADPH-consuming reaction of the auxiliary enzyme phloroglucinol reductase (PR) that was present in the cell extracts at nonlimiting activities. The extinction decrease owing to NADPH oxidation was recorded at 365 nm ($\varepsilon_{365} = 3.5\ \mathrm{mM^{-1}\ cm^{-1}}$; Ziegenhorn et al. 1976). The assay was carried out in rubber-stoppered glass cuvettes under an N_2 atmosphere at room temperature. The spectrophotometers used were model 100–40 (Hitachi, Tokyo, Japan) and Uvikon 860 (Kontron, Zurich, Switzerland). Anoxic handling of reagents has been described previously (Kreft and Schink 1994). The standard reaction mixture contained the following components (final concentrations) added in this sequence: 50 mM potassium phosphate (pH 7.2), 1 mM $MgCl_2$ (Ti^{3+} precipitates at higher Mg^{2+} concentrations), 10 mM ATP, 2.5 mM Ti^{3+} chelated by 3.75 mM nitrilotriacetate, 0.5 mM NADPH, 10 mM [6*RS*]-5,6,7,8-tetrahydrofolate (H_4folate), cell extract (2.5 mg ml^{-1}), and 10 mM methyl donor. Reagent stock solutions were prepared with 50 mM potassium phosphate (pH 7.2; except Mg^{2+}, ATP, and Ti^{3+}), and the pH of the stock solutions of ATP, Ti^{3+}, and H_4folate was adjusted to 7.2 by adding KOH (or NaOH to ATP). Titanium(III) nitrilotriacetate stock solutions (100 mM Ti^{3+} chelated by 150 mM nitrilotriacetate) were prepared in an anoxic chamber (Moench and Zeikus 1983). The reaction was started by the addition of the methyl donor (e.g., 3,5-dihydroxyanisole) about 10 min after addition of the cell extract. This preincubation was necessary for activation of the enzyme system (Kreft and Schink 1993). If CH_3SH was used as the methyl acceptor instead of H_4folate, the cell extract was added and excess pressure was released before gaseous CH_3SH was added to a final concentration of 5.8 mM in the liquid phase. Addition of gaseous CH_3SH, in contrast to CH_3SNa, did not change the pH or the Na^+ concentration. CH_3SH concentrations were calculated according to Henry's constant (1.79 atm l mol^{-1} at 25°C in distilled water; Dacey et al. 1984). Cuvettes with a 5-mm light path were used (400 µl liquid in 770 µl total volume) o allow use of high protein concentrations.

Phloroglucinol reductase assay

The enzyme was measured as described above, but with the following reaction mixture (final concentrations): 50 mM potassium phosphate (pH 7.2), 0.5 or 1 mM NADPH, cell extract (5–30 µg protein ml^{-1}), and 1 mM phloroglucinol. Phloroglucinol stock solutions were prepared in 50 mM potassium phosphate (pH 7.2), and the pH was adjusted to 7.2 with NaOH.

Discontinuous assays for demethylations and subsequent transformations

Assays were carried out as described above, but with 1 mM methyl donor and H_4folate and without NADPH. Samples were removed from reaction mixtures with syringes, injected into 4 parts 100 mM H_3PO_4 to terminate the reaction, and analyzed by HPLC.

Calculation of kinetic parameters

Kinetic equations were fitted to the data by a least-squares algorithm without linear transformation of the equations.

Analytical methods

Aromatic compounds and folates were analyzed by C_{18} reversed-phase HPLC [Beckman System Gold (Beckman Instruments, Munich, Germany) equipped with a Beckman Ultrasphere-ODS column, 4.6 × 150 mm] and photometric detection as described previously (Brune and Schink 1990). Folate specimens were handled anoxically, kept on ice, and analyzed soon after sampling. Protein concentrations were determined by an improved Bradford procedure (Read and Northcote 1981) with dye-reagent no. 1 and with ovalbumin as standard.

Chemicals

5-Hydroxyvanillate was purchased from Pfaltz & Bauer (Waterbury, Conn., USA), guaiacol and 3-methoxycatechol from Acros Chimica (Nidderau, Germany), and vanillic acid and isovanillic acid from Sigma (Deisenhofen, Germany). $TiCl_3$ (15%) in 10% HCl (ampoules), phloroglucinol, [6*RS*]-5,6,7,8-tetrahydrofolate 3HCl, and [6*RS*]-5-methyl-5,6,7,8-tetrahydrofolate (Ca salt) were obtained from Merck (Darmstadt, Germany); NADPH (Na-salt), ATP (Na-salt), and DNase I (EC 3.1.21.1) were from Boehringer (Mannheim, Germany); CH_3SH and most aromatic compounds were from Fluka (Neu-Ulm, Germany). Gases were of the highest commercial purity and were obtained from Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Growth with 3,5-dihydroxyanisole and substrate specificity of extracts

Holophaga foetida strain TMBS4 grew with 3,5-dihydroxyanisole ($\mu = 0.049\ h^{-1}$, $t_d = 14$ h) and fermented it completely to acetate. The growth yield was 13 g dry cell mass mol^{-1}; 3,5-dihydroxyanisole was stable under these culture conditions.

The specificity of demethylation activities was tested in cell-free extracts by measuring time courses of substrates, intermediates, and products by HPLC. Methylated pyrogallol and catechol derivatives were demethylated at

Table 1 Substrate specificity of extracts of cells grown with 3,4,5-trimethoxybenzoate or 3,5-dihydroxyanisole. Tetrahydrofolate (H_4folate; 2 mM) was used as methyl acceptor; formation of CH_3-H_4folate was analyzed by HPLC. Similar results were obtained with CH_3SH as methyl acceptor (the first eight compounds listed were tested). The protein concentration in the assays was 2.5 mg protein ml^{-1}. Reactions were started by addition of 2 mM methyl donor; all methyl donors were stable under assay conditions. Growth data have been published (Liesack et al. 1994) (*nt* not tested)

Methyl donor	Growth observed with donor	Relative demethylation activities in extracts of cells grown with:	
		Trimethoxy-benzoate	Dihydroxy-anisole
3,4,5-Trimethoxybenzoate	+	0.84	0.00
Syringate	+	0.92	0.09
5-Hydroxyvanillate	+	1.00[a]	0.07
3-Methoxycatechol	nt	0.68	0.10
Vanillate	–	0.95	0.07
Isovanillate	nt	0.96	0.03
Guaiacol	nt	0.77	0.07
3,5-Dihydroxyanisole	+	0.04	1.00[b]
3-Hydroxyanisole	nt	0.25	0.45
4-Hydroxyanisole	nt	0.03	0.09
Anisole	–	0.21	0.09
2-Methoxybenzoate	–	0.03	0.00
3-Methoxybenzoate	–	0.36	0.00
4-Methoxybenzoate	–	0.36	0.00

[a] The activty was 20 nmol min^{-1} ml^{-1}

[b] The activity was 41 nmol min^{-1} ml^{-1}

high rates by extracts of 3,4,5-trimethoxybenzoate-grown cells, but 3,5-dihydroxyanisole was not significantly demethylated (Table 1). Gallate and protocatechuate were decarboxylated by this cell extract to yield pyrogallol or catechol, respectively, both of which were not converted further. In contrast, extracts of 3,5-dihydroxyanisole-grown cells demethylated only 3,5-dihydroxyanisole and 3-hydroxyanisole at high rates (Table 1); gallate and protocatechuate were not decarboxylated.

Coupled photometric enzyme assay for demethylation

The NADPH-dependent phloroglucinol reductase (PR) present in the cell extract was used as indicator enzyme in a coupled enzyme assay for the phloroglucinol formed in 3,5-dihydroxyanisole demethylation (Fig. 1). Hence, the properties of PR that are relevant for this assay were studied.

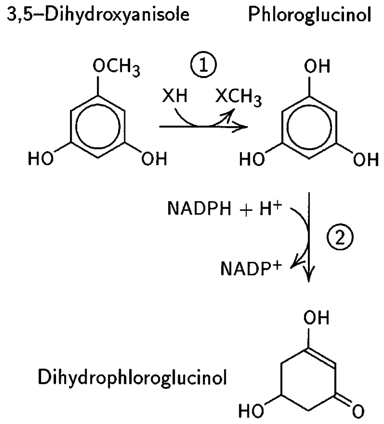

Fig. 1 The principle of the photometric coupled enzyme assay for demethylation. Demethylation of 3,5-dihydroxyanisole, the primary reaction (*reaction 1*), is catalyzed by the demethylating enzyme system of extracts of 3,5-dihydroxyanisole-grown cells with a methyl acceptor X [tetrahydrofolate (H_4folate) or CH_3SH]. The formation of phloroglucinol by the primary enzyme system is detected by the phloroglucinol-consuming and NADPH-oxidizing indicator reaction (*reaction 2*) that is catalyzed by phloroglucinol reductase. Several tautomeres of dihydrophloroglucinol can be envisaged; a comparison of literature data (Scott 1964) with the observed ultraviolet spectrum of dihydrophloroglucinol (λ_{max} = 258 nm in water, pH 2) supports the structure shown here

Properties of PR

Phloroglucinol was reduced to dihydrophloroglucinol as identified by HPLC on the basis of coelution and on-line ultraviolet spectra. The reaction was specific for NADPH (NADH could not substitute for NADPH) and depended on the simultaneous presence of cell extract, phloroglucinol, and NADPH. Accordingly, the 3,5-dihydroxyanisole demethylation reaction depended on the simultaneous presence of extract from phloroglucinol-grown cells, 3,5-dihydroxyanisole, one of the methyl acceptors tetrahydrofolate (H_4folate) or CH_3SH, and NADPH. The stoichiometry of phloroglucinol or 3,5-dihydroxyanisole and NADPH consumption was 1:0.95 ± 0.02 or 1:0.90 ± 0.01, respectively, as determined photometrically in three independent assays each. NADPH was the only reactant absorbing significantly at 365 nm; 3,5-dihydroxyanisole, phloroglucinol, dihydrophloroglucinol, H_4folate, CH_3-H_4folate, CH_3SH, and CH_3-S-CH_3 each had molar absorptivities lower than 1% of that of NADPH.

The specific activity of PR was 6,000 nmol min^{-1} (mg protein)$^{-1}$ at 1 mM phloroglucinol and NADPH, independent of protein concentration in the range of 5–30 μg protein ml^{-1}, and was stable for many days [decrease of specific activity: 6 nmol min^{-1} (mg protein)$^{-1}$ h^{-1}] if the cell extract was kept on ice. Exposure of cell extract to air for 30 min on ice had no significant effect on the activity of PR. Addition of 2–10 mM Mg^{2+}, ATP, Ti^{3+}, or CH_3SH had no significant effect on PR activity. However, PR activity decreased due to addition of H_4folate, combined addition of Mg^{2+}, ATP, and Ti^{3+}, or combined addition of Mg^{2+}, ATP, Ti^{3+}, and H_4folate by a factor of 0.58 ± 0.01, 0.77 ± 0.08, or 0.65 ± 0.04, respectively (n = 3). The enzyme did not reduce 3,5-dihydroxyanisole, as HPLC-analyzed controls proved. Commercial 3,5-dihydroxyanisole contained 0.27 mol% phloroglucinol; hence, at higher 3,5-dihydroxyanisole concentrations, a methyl-acceptor-independent extinction decrease due to phloroglucinol consumption was observed. Its rate and extent, however, were insignificant.

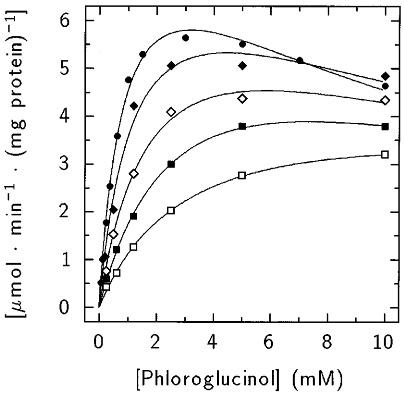

Fig. 2 Dependence of the specific activity of the indicator enzyme phloroglucinol reductase on the phloroglucinol concentration at 0.5 mM NADPH and various concentrations of the inhibitor 3,5-dihydroxyanisole in cell extracts. All curves are plots of substrate inhibition equations (Cornish-Bowden 1976) with the parameters given in Table 2. Reactions were started by addition of phloroglucinol. The protein concentration in the assays was 0.027 mg ml^{-1}. The concentrations of the inhibitor were: ● 0 mM, ◆ 10 mM, ◇ 25 mM, ■ 50 mM, and □ 100 mM

Table 2 Apparent kinetic constants of the indicator enzyme phloroglucinol reductase for phloroglucinol at 0.5 mM NADPH and various concentrations of the inhibitor 3,5-dihydroxyanisole. Reactions were started by addition of phlororglucinol. The protein concentration in the assays was 0.027 mg ml^{-1}. Plots of substrate inhibition equations with the parameters given here are shown in Fig. 2

Inhibitor concentration (mM)	K_m (mM)	K_{ss} (mM)	V_{max} [nmol min^{-1} (mg protein)$^{-1}$]	V_{max}/K_m [nmol min^{-1} (mg protein)$^{-1}$ mM^{-1}]
0	0.967	10.4	9,350	9,660
10	1.32	15.0	8,490	6,450
25	1.98	17.1	7,630	3,860
50	3.13	17.2	7,220	2,300
100	3.29	59.4	4,800	1,460

The specific PR activity depended on the concentration of phloroglucinol at various concentrations of the substrate-analogous inhibitor 3,5-dihydroxyanisole (Fig. 2; Table 2). These data allowed the calculation of the phloroglucinol consumption rate in all demethylation assays. The dependence of PR activity on phloroglucinol concentration exhibited substrate inhibition and followed the substrate inhibition equation (Cornish-Bowden 1976) at all inhibitor concentrations. Substrate inhibition – not the inhibition due to 3,5-dihydroxyanisol – was more pronounced (see the K_{ss} values and the stronger deviation from a Michaelis-Menten hyperbola) at lower 3,5-dihydroxyanisol concentrations and was observed whether or not the pH of the phloroglucinol stock solutions [prepared in 50 mM potassium phosphate (pH 7.2)] was adjusted to 7.2 with NaOH. The presence of the inhibitor 3,5-dihydroxyanisole decreased both the apparent (app.) V_{max} and app. V_{max}/app. K_m (the initial slopes of the curves in Fig. 2) and, hence, exhibited mixed inhibition (Cornish-Bowden 1976). By secondary plots (Cornish-Bowden 1976), the inhibition constants were estimated to be K_i = 18.4 mM (correlation coefficient 0.997) and K_i' = 107 mM (correlation coefficient 0.977) 3,5-dihydroxyanisole.

Effect of an additional supply of PR

In order to test the effect of doubling the already high PR activity in the standard coupled enzyme assay, we enriched PR from phloroglucinol-grown cells by anion exchange chromatography. The enriched fraction, displaying five bands in an SDS-PAGE gel, had a specific PR activity of 15 (mol min^{-1} (mg protein)$^{-1}$ (1 mM NADPH and phloroglucinol) and a protein concentration of 6.7 mg ml^{-1}; the PR activity did not decrease considerably during the experiments described below. The PR fraction had no demethylating activity with either methyl acceptor. The demethylation reaction in the coupled enzyme assays was not significantly affected by additions of the buffer present in the PR fraction; this buffer was nonetheless routinely added to the controls without PR additions. The demethylation reaction in the presence or absence of added PR depended only on the presence of one of the methyl acceptors. We doubled the PR activity of coupled enzyme assays with various concentrations of protein (1–3 mg protein ml^{-1} with either methyl acceptor), CH_3SH, H_4folate, or 3,5-dihydroxyanisole (with either methyl acceptor) by adding the PR-enriched fraction to reaction mixtures. The average of the demethylating activity without PR addition divided by the activity with PR addition was 1.0 ± 0.06 (in 18 independent assays).

Properties of 3,5-dihydroxyanisole demethylating enzyme activity

If H_4folate was used as the methyl acceptor for demethylation of 3,5-dihydroxyanisole or isovanillate, CH_3-H_4fo-

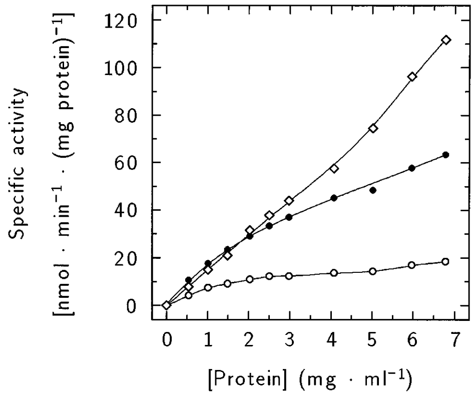

Fig. 3 Dependence of specific activity of 3,5-dihydroxyanisole demethylation on protein concentration. Methyl acceptors used were: ◇ 10 mM tetrhydrofolate (H_4folate), ○ 0.61 mM CH_3SH, and ● 5.8 mM CH_3SH. The curve for 15 mM CH_3SH was almost the same as the one for 5.8 mM and, hence, is not shown

Table 3 Apparent kinetic constants for 3,5-dihydroxyanisole demethylation. The protein concentration in the assays was 2.5 mg ml^{-1}. Reactions were started by addition of 3,5-dihydroxyanisole. The concentrations of the respective substrates that were not varied were: 1- mM 3,5-dihydroxyanisole, 10 mM tetrahydrofolate (H_4folate), and 22 mM CH_3SH (*na* not applicable)

Methyl acceptor	Substrate varied	K_m (mM)	V_{max} [nmol min^{-1} ml^{-1}]	V_{max}/K_m [nmol min^{-1} ml^{-1} mM^{-1}]
H_4folate	3,5-Dihydroxyanisole	< 0.01	105	na
CH_3SH	3,5-Dihydroxyanisole	< 0.01	84	na
H_4folate	H_4folate	0.79	100	130
CH_3SH	CH_3SH	1.7	100	60

late was formed as identified by HPLC on the basis of coelution and on-line ultraviolet spectra. The product of 3,5-dihydroxyanisole demethylation with either one of the methyl acceptors H_4folate or CH_3SH was phloroglucinol, as verified by HPLC using the same criteria as above.

The specific activity increased proportionally with protein concentration (Fig. 3). Note that the specific activity of the indicator enzyme was independent of protein concentration and in excess. With H_4folate as methyl acceptor, proportionality was found over an extended range of protein concentrations (Fig. 3). The in vivo activity of 110 nmol min^{-1} (mg protein)$^{-1}$, calculated with the measured growth rate and yield, was reached in vitro at the highest protein concentration used (about 7 mg ml^{-1}). With CH_3SH as methyl acceptor, we compared the effect of various CH_3SH concentrations (0.61 mM, 5.8 mM, and 15 mM) on the protein dependence of the specific activity (Fig. 3).

The dependence of 3,5-dihydroxyanisole demethylation rates on either [6*RS*]-H_4folate or CH_3SH concentrations followed Michaelis-Menten kinetics (Table 3). We attempted to measure the apparent Michaelis constant for 3,5-dihydroxyanisole in the demethylation reaction with H_4folate and CH_3SH as methyl acceptors (Table 3). The demethylation rate above 10 μM 3,5-dihydroxyanisole was more or less in the V_{max} or zero-order range; unfortunately, the assay was not suitable for measuring 3,5-dihydroxyanisole consumption rates at concentrations below 10 μM.

Discussion

Depending on the growth substrate, *Holophaga foetida* extracts contained either one of two demethylating enzyme systems with different specificities (or none at all if grown with gallate; Kreft and Schink 1993):

1. The *ortho* system had a broad specificity and reacted with aromatic compounds (not only growth substrates) that structurally resembled the growth substrate; it was present in extracts of 3,4,5-trimethoxybenzoate-grown cells. Enzymes with broad and similar specificity, recognizing substrates with at least two substituents in *ortho* position, were also found in *Clostridium thermoaceticum* and *Sporomusa ovata* (Daniel et al. 1991; Stupperich and Konle 1993).

2. The *meta* system displayed a novel specificity, reacting only with the two compounds with *meta*-hydroxyl groups, i.e., 3,5-dihydroxyanisole and 3-hydroxyanisole (extracts of 3,5-dihydroxyanisole-grown cells). *S. ovata* and *C. thermoaceticum* can also grow on a *meta* substrate, but only extracts of *ortho*-substrate-grown cells have so far been tested for demethylation of *meta* substrates (Daniel et al. 1991; Stupperich and Konle 1993). Both enzyme systems of *H. foetida* use the same methyl acceptors, require reductive activation, and display a quadratic dependence of activity on protein concentration. Also the gallate- and protocatechuate-decarboxylating activity was subject to induction and was present only in extracts of cells grown with the carboxylated substrate 3,4,5-trimethoxybenzoate.

The coupled photometric enzyme assay developed for demethylation reactions in vitro was suitable for initial rate measurements in cell extracts since downstream processes such as reduction of NADP$^+$ by 3-hydroxy-5-oxohexanoate dehydrogenase (Brune and Schink 1992; Kreft and Schink 1993) cannot influence initial rates (NADP$^+$ and 3-hydroxy-5-oxohexanoate are not present initially). With purified enzymes, this limitation of the assay is relieved, and reaction progress kinetics become meaningful at the same time.

At the beginning of the 3,5-dihydroxyanisole demethylation reaction, phloroglucinol starts to accumulate until it is removed by the indicator enzyme at the rate of its production. Thus, a short time interval is required for the system to reach steady-state. Since the rate of the indicator enzyme depends on the phloroglucinol concentration, this steady-state will be reached when the phloroglucinol concentration is high enough to allow an equally high activity of the indicator enzyme as long as the cosubstrate NADPH is in excess [see London and Shaw (1993) for calculations of lag time and steady-state concentrations]. The activity of the demethylating enzyme system will be limited by the activity of PR only if amounts of phloroglucinol that inhibit the forward reaction of demethylation (product inhibition; Cornish-Bowden 1976) have accumulated, or if the 3,5-dihydroxyanisole concentration has considerably decreased when the steady-state is reached. Examples of phloroglucinol concentrations during steady-state calculated according to London and Shaw (1983) and the appropriate parameters from Table 2 are 17 μM at the highest activity observed (Fig. 3) and 6 μM at the highest activities observed in substrate dependence measurements (Table 3). The pre-steady-state of increasing phloroglucinol concentration and PR activity was usually not observed (lag time always below 5 s), and the extent of 3,5-

dihydroxyanisole conversion during the lag phase (if observable) was insignificant (below approximately 5 μM) except for very low 3,5-dihydroxyanisole concentrations.

PR is an excellent indicator enzyme: it is specific at high and stable activity, is not sensitive to oxygen, and lacks special requirements. A disadvantage may be its inhibition by its substrate; however, this occurs only at unusually high concentrations of the substrate. PR is not present in other demethylating bacteria and has to be added for the test of purified enzymes as well. The reductase can be supplied by extracts of phloroglucinol-grown *H. foetida* or other anaerobic bacteria (Haddock and Ferry 1989; Brune and Schink 1992; Schink et al. 1992); PR has been purified from *Eubacterium oxidoreducens* (Haddock and Ferry 1989). We used a PR-enriched fraction to prove that the indicator enzyme did not limit demethylation rates, which is in accord with the above-mentioned calculations using the kinetic constants of the PR. The kinetic properties of PR of *H. foetida* were, as far as studied, similar to those of the enzyme of *E. oxidoreducens* (Haddock and Ferry 1989).

Another coupled enzyme assay for demethylation with a different – though overlapping – area of applicability was described by Meßmer et al. (1996). It is based on quantitation of CH_3-H_4folate by a set of three indicator enzymes and can be used for all tetrahydrofolate (H_4folate)-dependent reactions.

Specific 3,5-dihydroxyanisole-demethylating activity in *H. foetida* extracts increased with protein concentration with H_4folate or CH_3SH (Fig. 3). This increase cannot be attributed to the coupled enzyme assay, but proves that the indicator enzyme did not limit the rate of the primary reaction since the specific activity of PR was independent of protein concentration. With H_4folate as methyl acceptor, the in vivo activity was reached at a sufficient protein concentration. The specific rate of syringate demethylation with CH_3SH as methyl acceptor in extracts of 3,4,5-trimethoxybenzoate-grown cells likewise depends on protein concentration, indicating a multicomponent enzyme system as discussed previously (Kreft and Schink 1993, 1994). Each of the methyl acceptors controlled activity (Table 3) as described by the Michaelis-Menten equation: apparent V_{max} was the same for H_4folate and CH_3SH; the substrate specificity constant V_{max}/K_m (initial slope) for H_4folate was approximately twofold higher than for CH_3SH.

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