Evidence for the Existence of Independent Chloromethane- and S-Adenosylmethionine-Utilizing Systems for Methylation in *Phanerochaete chrysosporium*

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O-methylation of acetovanillone at 4 position by CH$_3$Cl and S-adenosyl[methyl-2H$_3$]methionine was monitored in whole mycelia of *Phanerochaete chrysosporium* in the presence and absence of S-adenosylhomocysteine. Both the amount of the methylation product, 3,4-dimethoxyacetophenone, and the percent CH$_3$Cl incorporation into the 4-methoxy group of the compound were determined. The results strongly suggest the presence of biochemically distinct systems for O-methylation of acetovanillone utilizing S-adenosylmethionine and chloromethane, respectively, as the methyl donor. The S-adenosylmethionine-dependent enzyme is induced early in the growth cycle, with activity attaining an initial maximum after 55 h of incubation. Methylation by this enzyme is totally suppressed by 1 mM S-adenosylhomocysteine over almost the entire growth cycle. S-Adenosylmethionine-dependent O-methyltransferase activity is detectable in cell extracts, and the purification and characterization of the enzyme are described elsewhere (C. Coulter, J. T. Kennedy, W. C. McRoberts, and D. B. Harper, Appl. Environ. Microbiol. 59:706–711, 1993). The chloromethane-utilizing methylation system is absent in early growth but attains peak activity in the mid-growth phase after 72 h of incubation. The system is not significantly inhibited by S-adenosylhomocysteine at any stage of growth. No chloromethane-dependent O-methyltransferase activity is detectable in cell extract, suggesting that the enzyme is membrane bound and/or part of a multienzyme complex. Although the biochemical role of the chloromethane-dependent methylation system in metabolism is not known, one possible function could be the regeneration of veratryl alcohol degraded by the attack of lignin peroxidase.

Chloromethane (CH$_3$Cl), a gaseous natural product released by many species of the family *Hymenochaetaceae*, a widespread family of wood-rotting bracket fungi, has a metabolic role in the biosynthesis of methyl esters of aromatic acids by these fungi (11, 15). The utilization of CH$_3$Cl as an endogenous methyl donor is confined to fungi which emit CH$_3$Cl but can occur in other widely studied lignin-degrading fungi such as *Phanerochaete chrysosporium*, *Coriolus versicolor*, and *Phlebia radiata*, none of which releases gaseous CH$_3$Cl at any stage of growth (9). Presumably, such fungi possess a tightly channelled system in which CH$_3$Cl biosynthesis is so closely coupled to utilization that release of gaseous CH$_3$Cl never occurs. In these species, CH$_3$Cl has been shown to act as a precursor of the methoxyl groups of veratral alcohol (3,4-dimethoxybenzyl alcohol) (9), a secondary metabolite believed to have a key role in fungal lignin degradation. Veratral alcohol has been reported to increase the level of ligninolytic enzymes in *P. chrysosporium* (6, 8, 14) and to be a substrate for lignin peroxidase, which is a major component of the fungal ligninolytic system (2, 13). The oxidation of monomethoxy- and aromatic compounds, normally poor substrates for lignin peroxidase, is stimulated by veratral alcohol, and it has been postulated that the compound may behave as a 1-electron redox mediator in their oxidation and in lignin depolymerization (12). Veratral alcohol may also prevent inactivation of lignin peroxidase (7, 17).

The extent to which CH$_3$Cl participates in methylation reactions in white rot fungi is still far from clear, and the biochemical rationale for its utilization in preference to the more conventional methylating agent S-adenosylmethionine (SAM) in some metabolic processes has yet to be delineated. While the ultimate origin of the methyl group of CH$_3$Cl is now well established as the amino acid methionine (10, 18), it has yet to be resolved whether the metabolic pathway from the compound involves SAM itself.

Here we report investigations designed to determine the relative utilization of CH$_3$Cl and S-adenosyl-[methyl-2H$_3$]methionine ([methyl-2H$_3$]SAM) as methyl donors in methylation of the synthetic substrate acetovanillone in whole cells and in cell extracts of *P. chrysosporium* at different stages of growth. Acetovanillone (3-methoxy-4-hydroxyacetophenone) is known to be readily methylated in the 4 position by ligninolytic cultures of *P. chrysosporium* (3) and has been shown to be a substrate for a SAM-dependent O-methyltransferase recently isolated from the organism (4). Methylation of acetovanillone by the labelled methyl donors both alone and in combination was monitored in the presence and absence of S-adenosylhomocysteine (SAH), which is a powerful competitive inhibitor of the SAM-dependent O-methyltransferase (4). The suppression of the SAM system by the use of SAH in this way allowed the relative contributions of SAM and CH$_3$Cl to overall acetovanillone methylation to be accurately assessed at each stage of growth.

**MATERIALS AND METHODS**

*Culture of fungus.* *P. chrysosporium* INA-12 (CN5M1-398) was a strain utilized by Harper et al. (9). The fungus was
grown without agitation at 37°C in 2-liter-volume conical flasks in 200 ml of medium which contained the following (in grams per liter): KH₂PO₄, 0.2; MgSO₄, 7H₂O, 0.05; NaCl, 0.59; CaCl₂·2H₂O, 0.013; thiamine, 0.0025; yeast extract (Difco), 0.1; glyc erol, 10; L-asparagin e monohydrate, 1.0; NH₄NO₃, 0.5; and 2,2-dimethylsuc cinic acid, 1.46. It also contained 1 ml of trace element solution per liter (1). The medium was adjusted to pH 5.0 with 2 M KOH, filter sterilized, and inoculat ed with approximately 5 × 10⁶ conidia per ml prepared as described by Eriksson and Johnsrud (5). Flasks were flushed with 100% O₂ for 2 min before being stoppered with sterile polytetrafluoroethylene-coated rubber stoppers. Cultures were harvested by filtration after periods of incubation ranging from 24 to 101 h. Superficial moisture was removed by pressing mycelia between filter paper, and the mycelia were either used directly in the standard assay of methylating activity or frozen at −15°C prior to preparation of cell extracts.

Assessment of growth. Mycelial growth was measured at various stages of incubation as the weight of mycelia harvested by filtration and dried at 100°C.

Assay of CH₃Cl- and SAM-dependent methylating activities. Mycelia (0.1 g) or cell extract (0.5 ml) at each stage of growth was incubated at 37°C in triplicate 40-ml-volume screw-cap septum vials lined with polytetrafluoroethylene-coated silicone discs. The vials contained, in a total volume of 2 ml, 2,2-dimethylsuccinate buffer (pH 6.5) (50 mM), acetovanillone (0.5 mM), and the labelled or unlabelled methyl donor (0.5 mM) or a combination of the two methyl donors (0.5 mM each). When chloromethane was utilized as the methyl donor, the concentration referred to above is that after equilibration of the gaseous and aqueous phases in the vial. The following combinations of methyl donor were used in the assay: CH₃Cl, [methyl-²H₃]SAM, SAM, CH₃Cl plus [methyl-²H₃]SAM, CO₂HCl plus SAM, CH₃Cl plus SAM, and no added methyl donor. The experiment was conducted in both the presence and the absence of 1 mM SAH. After 2 h of incubation, the reaction was terminated by quickly freezing the mixture at −15°C and storing it at this temperature until extraction and quantitation of 3,4-dimethoxyacetophenone (DMAP) and determination of percent CH₃H₂ incorporation into the compound. In initial time course studies, the incubation time ranged from 15 min to 6 h. Cell extracts prepared from mycelia at each stage of growth were assayed as described above, except that mycelia were replaced by cell extract (0.5 ml). All experimental values given are the means of three replicates. The whole experiment was repeated three times, and a similar pattern was observed in each case. The results given in this paper are those of one representative experiment.

Preparation of cell extracts. Frozen mycelia (5 g) were suspended in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM dithiothreitol (5 ml) and macerated with a tissue grinder attached to a Black and Decker power drill at maximum speed for a period of 10 min. Cooling in an ice bath ensured that the temperature did not rise above 4°C. The resulting homogenate was centrifuged at 50,000 × g for 30 min at 4°C, and the clear supernatant (7.5 ml) was decanted.

Determination of DMAP and percent incorporation of CH₃H₂ into DMAP. The assay mixture (2 ml) after the addition of 4-methoxyacetophenone (0.2 ml of 50 μg/ml of solution in acetone) as an internal standard was extracted three times with chloroform (10 ml per extraction), and the bulked extract was washed with saturated NaCl solution (5 ml). The extract was dried over anhydrous MgSO₄ and evaporated to 0.5 ml under reduced pressure. Finally, the solution was brought to 1 ml with diethyl ether.

DMAP was determined by gas chromatography-mass spectrometry on a Hewlett Packard 5890 gas chromatograph linked to an HP5970 mass selective detector controlled by an HP300 series computer. The gas chromatograph was fitted with an Ultra 1 fused silica wall-coated open tubular capillary column (25 m by 0.2 mm) with 100% dimethylpolysiloxane as the bonded phase. Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹, and a split ratio of 20:1 was employed. After injection of the sample, the oven temperature was maintained at 100°C for 1 min and then programmed at 10°C min⁻¹ up to 160°C followed by 30°C min⁻¹ up to 300°C. To quantify DMAP, the ion currents at m/e 180 and 183 were monitored and their sum at the retention time of the compound was compared with the ion current at m/e 180 given by an authentic sample of the compound. The ion current at m/e 150 (the molecular ion of the internal standard) was also monitored to allow appropriate adjustment to be made for incomplete recovery of DMAP. The incorporation of CH₃H₂ into the 4-O-methyl group of DMAP was calculated as the ratio of ion currents m/e 183/(m/e 180 + m/e 183) at the retention time of the compound.

Chemicals. CH₃H₂Cl (99.9 atom% ²H) and S-adenosyl-l-[methyl-²H₃]methionine (99.5 atom% ²H) as the p-toluene sulfonate salt were obtained from MSD Isotopes, Montreal, Quebec, Canada. Chloromethane was purchased from BDH, Poole, Dorset, United Kingdom, and S-adenosyl-l-methionine p-toluenesulfonate salt and S-adenosyl-l-homocysteine were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom. Acetonilavonilone, 4-methoxyacetophenone, and DMAP were provided with Aldrich Chemical Co., Gillingham, Dorset, United Kingdom. N-Methyl-N-trimethylsilyl trifluoroacetamide reagent was obtained from Pierce Chemical Co., Rockford, Ill.

RESULTS

Time course and pH dependence of acetovanillone methylation in whole mycelia. The development of a reliable assay for SAM-dependent and CH₃Cl-dependent acetovanillone methylation activities in whole mycelia necessitated an initial investigation of the time course and pH dependence of methylation by both methyl donors. Figure 1 shows SAM- and CH₃Cl-dependent methylation in mycelia of P. chrysosporium harvested after 60 h of growth and assayed at pH 6.5. Endogenous methylation in the absence of added methyl donor is also recorded. It is clear that the rate of methylation is linear over at least 4 h in the presence of both CH₃Cl and SAM. CH₃Cl-dependent methylation cannot be attributed to a stimulation of endogenous SAM-dependent methylation by CH₃Cl, since when the experiment was conducted with CH₃Cl, the methylated product, DMAP, exhibited high levels of CH₃H₂ incorporation. After 15 min of incubation, 62% CH₃H₂ incorporation was observed, and a plateau value of 70% was attained within 1 h.

The pH optimum for SAM-dependent methylation by whole mycelia was found to be 7.5 to 8.0, a value consistent with the pH optimum (pH 7 to 9) shown by the SAM-dependent O-methyltransferase previously purified from P. chrysosporium (4). In contrast, the optimum pH for CH₃Cl-dependent methylation, pH 6.0, was significantly lower. As it was considered vital that, in further comparative experiments with the two systems in whole mycelia, the relative activities should be measured under identical conditions, a pH of 6.5 which was intermediate between the optima of the
systems was chosen for routine assay of both SAM- and CH₃Cl-dependent methylation. At this compromise pH, the CH₃Cl-dependent system possessed 60% of the activity displayed at its pH optimum, while the SAM-dependent system manifested 50% of the activity exhibited at its pH optimum.

Growth and 4-O-methylation activity in whole mycelia. Acetovanillone-methylating activity was measured at various stages of growth with different ²H-labelled methyl donors. Growth of the fungus, measured as mycelial dry weight, is shown in Fig. 2, and the changes in SAM- and CH₃Cl-dependent methylating activities are recorded in Fig. 3a. The experiment was also performed in the presence of identical concentrations of unlabelled methyl donors, but as no significant differences were observed in these methylating activities compared with those of the labelled compounds, only the results for the labelled compounds are recorded in Fig. 3a. Methylating activities were also measured in the presence of 1 mM SAH (Fig. 3b). This compound had previously been shown to be a powerful competitive inhibitor of the SAM-dependent O-methyltransferase present in P. chrysosporium, with a Kᵢ of 41 μM (4). Figure 4a and b record the incorporation of C₂H₃ into the 4-O-methyl group of DMAP at formed in the experiments shown in Fig. 3a and b.

Figure 3a clearly shows the activity of the SAM-dependent methylating system reaching a peak after 55 h of incubation, coincident with the early exponential growth phase of the fungus. Activity then falls slightly over the next 20 h before it rises again as the fungus approaches the stationary phase and sporulation commences. In contrast, CH₃Cl-dependent methylation attains a somewhat lower maximum about 20 h later than the SAM-dependent peak, coincident with the mid-growth phase of the fungus. Activity remains at this plateau value for about 30 h before it falls as the fungus enters the stationary phase. After 70 h of growth, when the CH₃Cl-dependent system is at maximum activity, the relative rates of methylation displayed by the SAM- and CH₃Cl-dependent systems assayed at pH 6.5 are 200 and 170 μmol/g (fresh weight) of mycelium per h, respectively, or, when corrected to the pH optimum for each system, 400 and 283 μmol/g (fresh weight) of mycelium per h, respectively.

The levels of C₂H₃ incorporation into the 4-O-methyl group of DMAP at C-4 from both [methyl-²H₃]SAM and C₂H₃Cl during growth lend support to the theory that CH₃Cl- and SAM-dependent methylation systems are quite distinct biochemically (Fig. 4a). High levels of C₂H₃ incorporation from [methyl-²H₃]SAM were measured during the early stages of growth, when the activity of the SAM-utilizing system was high. However, incorporation fell dramatically as the activity of the endogenous CH₃Cl-utilizing system increased, effectively enhancing the ratio of unlabelled to labelled methyl donor used in the cell. Conversely, C₂H₃ incorporation from C₂H₃Cl was low during early growth and rose to a maximum of more than 60% after 72 h, exhibiting a strong correlation with the activity of the CH₃Cl-dependent methylating system. The C₂H₃ incorporation observed from a C₂H₃Cl-[methyl-²H₃]SAM mixture remained high throughout growth but did not attain 100%, a finding explicable in the terms of label being diluted by the presence of unlabelled product generated from endogenous methyl sources, probably mainly CH₃Cl, in view of the background methylation pattern shown in Fig. 3a. The apparent availability of endogenous CH₃Cl also explains why the level of C₂H₃ incorporation in the presence of exogenous C₂H₃Cl alone never reached the level achieved in the presence of [methyl-²H₃]SAM alone.

The activity of the SAM-dependent methylating system was almost completely inhibited by SAH, with the amount of DMAP formed at all stages of growth being indistinguishable from the background levels of methylation activity in the absence of added methyl donor (Fig. 3b). In contrast, the presence of SAH had very little effect on acetovanillone methylation by the CH₃Cl-utilizing system. Indeed, the maximum rate of CH₃Cl-dependent methylation was approximately 13% greater in the presence of SAH. When the C₂H₃Cl-[methyl-²H₃]SAM combination was employed as
the methyl donor, the presence of SAH caused a significant decrease in the rate of methylation and the levels of DMAP formed became virtually identical to those observed when C2H3Cl alone was present as the methyl donor.

The selective inhibition of the SAM-dependent system by SAH is even more graphically illustrated by the effect of the compound on the C2H3 incorporation patterns. In the presence of the inhibitor, the incorporation from [methyl-2H3]SAM fell from 85 to 15% at 41 h, and it was completely eliminated at later stages of growth. In contrast, the maximum C2H3 incorporation level from endogenous C2H3Cl observed in the mid-growth phase was not significantly altered by SAH, and, indeed, the percent C2H3 incorporation was higher in early growth and in the late-exponential phase as a result of C2H3Cl supplanting SAM as the source of methyl groups for acetovanillone methylation. In accordance with these observations, C2H3 incorporation from the C2H3Cl-[methyl-2H3]SAM combination fell substantially in the presence of SAH compared with that observed with [methyl-2H3]SAM alone to become virtually identical over most of the growth cycle to that found with C2H3Cl alone.

Isotopic incorporations into DMAP after incubation of mycelia at various growth stages with a C2H3Cl-SAM or C2H3Cl-[methyl-2H3]SAM combination as the methyl donor were also examined (Fig. 5). In both the presence and absence of SAH, the general patterns of incorporation from C2H3Cl-SAM and CH3Cl-[methyl-2H3]SAM were similar to those observed when C2H3Cl and [methyl-2H3]SAM, respectively, were utilized separately as methyl donors. However, the enhanced product formation associated with the presence of the unlabelled alternative methyl donor led inevitably to a significant decrease in the extent of incorporation. In the absence of SAH, incorporation from CH3Cl-[methyl-2H3]SAM was initially high at 88% during the early growth phase, when the SAM-dependent but not the CH3Cl-dependent methylation system was active. Predictably, the subsequent decline in C2H3 incorporation as the latter system developed was more pronounced than that with [methyl-2H3]SAM alone as the methyl donor. C2H3 incorporation from the C2H3Cl-SAM combination was at all stages of growth lower than that obtained with C2H3Cl alone, reflecting the fact that even at peak activity of the CH3Cl-utilizing system, there is still substantial SAM-dependent methylating activity present in the mycelia. Nevertheless, the reciprocal relationship between the incorporation patterns obtained with the two methyl donor combinations is clearly apparent, and the experiment provides unambiguous confirmation of the peak in activity of the CH3Cl-dependent methylation
system at 72 h. When the percents incorporation found with the two methyl donor combinations were summed, overall incorporation was almost identical at all stages of growth to that shown in Fig. 4a for the C2H3Cl- [methyl-3H]SAM combination, an observation providing convincing evidence of the internal consistency of the experimental results. In the presence of SAH, no C2H3 incorporation from CH3Cl- [methyl-3H]SAM was detected but a dramatic increase in the percent C2H3 incorporation from C2H3Cl-SAM was recorded, with levels found being very similar to those noted with C2H3Cl alone, confirming the conclusions from Fig. 4b and 5b that the SAM-dependent methylating activity is completely suppressed by SAH, while CH3Cl-dependent activity is unaffected.

**4-O-methylation activity in cell extracts.** Cell extracts of mycelia harvested at various stages of growth were assayed for 4-O-methyltransferase activity at C-4 utilizing acetovanillone as the substrate and either C2H3Cl or SAM as the methyl donor. There was no evidence of CH3Cl-dependent methylation in such extracts at any stage of growth, implying that the system either is highly labile, perhaps membrane bound, or requires an as-yet-unidentified cofactor. On the other hand, substantial SAM-dependent methyltransferase activity was present in cell extracts after 41 h and increased over the following 30 h, briefly levelling off at 72 h before rising again in the late growth phase (Fig. 6). A comparison of Fig. 6 with Fig. 3a demonstrates that O-methyltransferase activity in cell extracts in general correlates quite well with SAM-dependent methylation in whole mycelia, although in the cell extracts the initial peak of activity during early growth seems much less pronounced than and somewhat delayed compared with that observed with whole mycelia. However, it must be borne in mind that the activity of the SAM-utilizing methylation system is critically dependent on the ratio of SAM to SAH. The decline in the activity of the system in whole mycelia between 52 and 72 h may be a reflection of an increase in the intracellular pool of SAH at a time when the rate of uptake of SAM (and hence the intracellular concentration) remained relatively constant. These circumstances would lead to a decrease in the ratio of SAM to SAH in the cell and an apparent fall in the SAM-dependent methylation activity recorded. In contrast, in cell extracts the concentration of SAM remains high, ensuring that despite possible increases in SAH concentration, inhibition remains minimal.

**DISCUSSION**

The results reported here provide compelling evidence for the existence of two biochemically distinct systems for O methylation of acetovanillone by *P. chrysosporium*, one route utilizing SAM as methyl donor and the other utilizing CH3Cl as the methyl donor. The enzyme utilizing SAM is induced early in the growth cycle. Its activity attains an initial maximum at 55 h in the early-exponential phase before declining slightly in the mid-growth phase and then rising again as sporulation commences in the late-growth phase. This SAM-dependent O-methyltransferase is active in cell extracts from which the enzyme has been purified and characterized, as reported by Coulter et al. (4), who showed that it could attack a range of 2,4-disubstituted phenols and that it was strongly inhibited by SAH. The latter observation suggested the use of SAH as a biochemical probe for the identification of SAM-dependent methylation in whole mycelia. In the experiments described in this paper, complete suppression of SAM-dependent methylation over almost the
entire growth cycle was indeed achieved by incubation in the presence of the compound. The CH₃Cl-utilizing methylation system is absent in early growth but attains peak activity in the mid-growth phase at 72 h before declining during the latter part of the growth cycle. No CH₃Cl-dependent O-methyltransferase activity could be detected at any stage of growth in cell extracts, implying that the enzyme either is highly labile or is not soluble. There are indications from previous work with the fungus Phellinus pomaceus that CH₃Cl biosynthesis and CH₃Cl-utilizing enzymes are associated in a membrane-bound multienzyme complex (15, 16). Unlike the SAM-dependent O-methyltransferase, the CH₃Cl-dependent enzyme is not inhibited by SAH in whole mycelia, and even complete suppression of the SAM-dependent O-methyltransferase by the compound does not appear to be associated with a significant change in CH₃Cl-dependent activity. This finding eliminates the possibility that CH₃Cl is converted to SAM prior to use in methylation unless a second, tightly channelled, SAM-utilizing methylation system exists which is not subject to inhibition by SAH and uses only SAM generated from CH₃Cl. However, as previous work on P. pomaceus has demonstrated that CH₃Cl, like SAM, originates from the amino acid methionine (10, 18), there would seem to be little biochemical rationale for such a circuitous metabolic route. It is perhaps significant that the presence of SAH has little effect on the background level of methylation in the absence of an exogenous methyl donor, suggesting that endogenous CH₃Cl is responsible for most of the methylation occurring in such circumstances. The function in metabolism of CH₃Cl-dependent methylation is at present far from clear. One possibility currently under examination is that a CH₃Cl-dependent O-methyltransferase is involved in the regeneration of veratryl alcohol, which in its role as a 1-electron redox mediator is continually being degraded by oxidation and demethylation by lignin peroxidase. It is essential that the compound is recycled efficiently by the fungus, since the energy cost of continual de novo biosynthesis would be exorbitant. Investigations now under way on the substrate specificity of the system in whole mycelia may shed light on this intriguing area.

REFERENCES