Methylation of phenol by chloromethane in the fungus *Phellinus pomaceus*

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The kinetics of phenol methylation by chloromethane (CH₃Cl) in intact mycelia of the fungus *Phellinus pomaceus* were examined. Anisole was produced linearly with respect to time at a rate of 6.3 nmol g⁻¹ h⁻¹ over 16 h when washed mycelia were incubated with phenol and C²H₃Cl. Incorporation of C²H₃- label into anisole attained a plateau value of 27% within 2 h. The rate of anisole production under N₂ was only 36% of that in air, and the proportion of C²H₃- label incorporated from exogenous C²H₃- increased from 30 to 45% under these conditions. The rate of methylation attained a maximum in 10 mM-phenol, but the level of activity was only about 10% of that of the fungal carboxyl-methylating system. The presence of exogenous C²H₃Cl clearly stimulated anisole formation, demonstrating that the rate of CH₃Cl biosynthesis limited methylation to some extent. In contrast to the carboxyl-methylating system, no significant inhibition of methylation was observed at methyl acceptor concentrations up to 17.5 mM, and no sharp fall in the rate of gaseous C²H₃Cl release or rapid increase in C²H₃-incorporation were observed at supraoptimal concentrations of acceptor, indicating that endogenous CH₃Cl biosynthesis was not inhibited under these conditions. Investigations of the rate of C²H₃-incorporation into anisole from exogenous C²H₃Cl showed a linear relationship between the logarithm of % C²H₃- incorporation and the logarithm of C²H₃Cl concentration, suggesting that, as postulated for the carboxyl-methylating system, the CH₃Cl-synthesizing and CH₃Cl-utilizing enzyme systems were situated on either side of a membrane within the cell.

Introduction

Gaseous chloromethane (CH₃Cl) is released during the idiophase by mycelia of most species of the Hymenochaetae, a widespread family of wood-rotting fungi (Harper, 1985; Harper & Kennedy, 1986; Harper *et al.*, 1988). The compound is produced in particularly high yield by species of the genus *Phellinus*, many of which also biosynthesize methyl esters of benzoic, salicylic and furoic acids as natural products (Harper *et al.*, 1988). Using C²H₃-labelled methionine, Harper & Hamilton (1988) showed that ester formation and CH₃Cl biosynthesis were biochemically linked in *Phellinus pomaceus*. These investigations culminated in the discovery that CH₃Cl acts as a methyl donor in methylation of benzoic and furoic acids during primary metabolism of the fungus (Harper *et al.*, 1989). The kinetics of the system in intact mycelia with reference to both benzoic acid and the non-physiological substrate butyric acid have been defined by McNally *et al.* (1990).

Methylation of compounds other than carboxylic acids by CH₃Cl was also observed in *P. pomaceus* (Harper *et al.*, 1989). A CH₃Cl-utilizing system capable of methylat-

ing a variety of substituted phenols and thiophenol has been identified in the fungus. Although the system displayed some activity in the growth phase, maximum activity was associated with the idiophase, when a sudden fourfold increase occurred, clearly demonstrating that the system was biochemically distinct from the carboxylic acid-methylating system which attains maximum activity in mid growth phase. Levels of C²H₃-incorporation from exogenous C²H₃Cl into anisole were generally lower than those into methyl ester suggesting an even tighter channelling of CH₃Cl during phenol methylation than that observed during carboxylic acid-methylation. Although the natural substrate of the phenol-methylating system has yet to be identified, the attainment of maximum activity in the idiophase implies that the enzyme is involved in secondary metabolism. In this context it is perhaps significant that a recent report by Harper *et al.* (1990) suggests that CH₃Cl participates in the biosynthesis of veratryl alcohol, a key idiolite produced by several species of white rot fungi in the Corticaceae and Polyporaceae, and in particular by *Phanerochaete chrysosporium* (Lundquist & Kirk, 1978). Veratryl alcohol induces components of the ligninolytic

system (Faison & Kirk, 1985; Faison *et al.*, 1986; Leisola *et al.*, 1984), prevents inactivation of ligninase (Hammerli *et al.*, 1986b; Tonon & Odier, 1988) and is reported to enhance the oxidation of lignin by lignin peroxidase *in vitro* (Hammerli *et al.*, 1986a). It may also function as a one-electron redox mediator in the depolymerization process, possibly promoting oxidation by the creation of activated oxygen species (Harvey *et al.*, 1986; Schoemaker *et al.*, 1985). Although veratryl alcohol has not been reported as occurring in *P. pomaceus* or indeed any species of the Hymenochaetaceae, the phenol-methylating system present in *P. pomaceus* may be involved in the biosynthesis of methoxylated aromatic secondary metabolites with an analogous role to that of veratryl alcohol in lignin degradation by *P. chrysosporium*.

In this paper we describe further investigations of the phenol-methylating system in intact mycelia of *P. pomaceus*, with particular reference to the effect of C^2H_3Cl and phenol concentration on the rate of methylation and the incorporation of C^2H_3 - into anisole. The effect of phenol concentration on release of CH_3Cl by mycelia has also been examined. The results of these studies are compared with those reported for the carboxylic acid-methylating system by McNally *et al.* (1990).

Methods

Organisms and maintenance media. *Phellinus pomaceus* (Pers.) Maire (NCWRF-FPRL 33A) was used in this study and maintained as described by McNally *et al.* (1990).

Chemicals. C^2H_3Cl (99.9 atom% 2H) was purchased from MSD Isotopes, Montreal, Canada. Chloromethane and agarose '10' Electran were obtained from BDH, and anisole was acquired from Aldrich.

Culture media and cultural conditions. The fungus was grown on glucose/mycological peptone/agarose medium overlaid with cellulose membrane as described by Harper *et al.* (1989).

Assay of methylating activity. Measurement of the rate of methylation of phenol by *P. pomaceus* and the percentage C^2H_3 - label incorporated into anisole was based on the standard assay procedure described by Harper *et al.* (1989) using 1 mM-phenol as substrate and 1.23 mM- C^2H_3Cl as methyl donor. In some experiments the concentrations of phenol and C^2H_3Cl were varied or the assay conducted under N_2 . In time-course studies, the assay was initiated by simultaneous injection of phenol and C^2H_3Cl into the vial, and incubation periods of 1 to 16 h were used. In all experiments, except those where standard deviations are quoted, assays were conducted in triplicate and the mean methylating activity expressed in nanomoles of anisole formed per g fresh wt of mycelium per hour ($nmol\ g^{-1}\ h^{-1}$). Where the standard deviations of results are given, five replicates were employed.

Measurement of CH_3Cl production by isolated mycelia. Production of CH_3Cl by mycelia suspended in citrate buffer in the presence of phenol was measured by gas chromatography using a headspace technique (McNally *et al.*, 1990).

Determination of anisole and of incorporation of C^2H_3 - into methoxyl group. Anisole formed in the assay solution was determined by a headspace technique. The solution (1 ml) was placed in a 20 ml vial

sealed with a crimped cap and septum lined with PTFE. The headspace in the vial was analysed by gas chromatography/mass spectrometry using conditions identical to those described by McNally *et al.* (1990) for the analysis of methyl benzoate. The Mass Selective Detector was operated in the selected ion-monitoring mode, and the sum of the ion currents at m/e 108 and m/e 111 at the retention time of anisole was compared to a calibration graph obtained using authentic compound. The percentage incorporation of C^2H_3 - was measured as the ratio of ion currents

$$\frac{m/e\ 111}{m/e\ 108 + m/e\ 111}$$

Results and Discussion

Time course of methylation in washed mycelia

Production of anisole with respect to time was linear for at least 16 h when mycelia of *P. pomaceus* were incubated with 1 mM-phenol in the presence of 1.23 mM- C^2H_3Cl (Fig. 1). The C^2H_3 - label from exogenous C^2H_3Cl was rapidly incorporated into the methoxyl group of anisole, attaining a maximum value of about 27% within 2 h, and thereafter remaining at approximately this level for the remainder of the incubation period. The rapid achievement of a plateau level of labelling mirrors the results obtained by McNally *et al.* (1990) in studies of the carboxylic acid-methylating system in the fungus, and confirms their conclusion that it is unlikely that CH_3Cl undergoes conversion to a freely diffusible intermediate before acting as a substrate for the methylating system. Nevertheless, it is possible to envisage a mechanism in which a protein-bound intermediate is formed by alkylation of a thioether by CH_3Cl to form a sulphonium derivative which then acts as methyl donor for the methylation of the phenolic hydroxyl.

When mycelia were incubated for 12 h with 1 mM-phenol under standard assay conditions in the presence of nitrogen, instead of air, the rate of anisole formation fell from $8.4 \pm 1.0\ nmol\ g^{-1}\ h^{-1}$ (mean $\pm$ SD) in air to $3.1 \pm 0.7\ nmol\ g^{-1}\ h^{-1}$ under nitrogen, and the C^2H_3 - label in the anisole increased from $30 \pm 3\%$ to $46 \pm 6\%$. This effect can be attributed, as with the carboxylic acid-methylating system (McNally *et al.*, 1990), to the known sensitivity of endogenous CH_3Cl biosynthesis to oxygen limitation, rather than an effect on the methylating system *per se*.

Effect of phenol concentration on anisole biosynthesis, C^2H_3 - incorporation into anisole from exogenous C^2H_3Cl , and CH_3Cl release

The influence of phenol concentrations up to 17.5 mM on anisole formation by washed mycelia of *P. pomaceus* in the presence and absence of 1.23 mM- C^2H_3Cl is shown in Fig 2(a). The rate of methylation was maximal at

Fig. 1. Effect of incubation time on phenol methylation and incorporation of $C^2H_3^-$ into anisole from exogenous C^2H_3Cl in washed mycelia of *P. pomaceus*. Washed mycelia were incubated as described in Methods with 1 mM-phenol and 1.23 mM- C^2H_3Cl . Anisole formation and incorporation of $C^2H_3^-$ into anisole were measured at intervals over 16 h. ○, Anisole production; ●, percentage of anisole labelled with $C^2H_3^-$.

Fig. 2. Effect of phenol concentration in washed mycelia of *P. pomaceus* on (a) anisole formation in the presence and absence of C^2H_3Cl ; and (b) incorporation of $C^2H_3^-$ label into anisole and release of CH_3Cl . Washed mycelia were incubated for 12 h as described in Methods with different concentrations of phenol in the presence and absence of 1.23 mM- C^2H_3Cl . Anisole production and incorporation of $C^2H_3^-$ into anisole were measured. In a separate experiment, mycelia of the same origin were incubated for 12 h (as described in Methods) in 20 mM citrate buffer, pH 4.0, with different concentrations of phenol, and CH_3Cl released by the mycelia was measured by gas chromatography. □, Anisole production in absence of exogenous C^2H_3Cl ; ○, anisole production in presence of 1.23 mM- C^2H_3Cl ; ●, percentage of anisole labelled with $C^2H_3^-$; △, CH_3Cl released by mycelia.

concentrations above 10 mM, but the level of activity of the system was only about 10% of that of the carboxylic acid-methylation system reported by McNally *et al.* (1990); however, this comparatively low rate must be viewed in the context that phenol is almost certainly not the natural substrate of the system. The presence of C^2H_3Cl clearly stimulated anisole formation, indicating that as with the methylation of benzoic acid by the carboxylic acid-methylating system (Harper *et al.*, 1989; McNally *et al.*, 1990), the endogenous rate of CH_3Cl biosynthesis limits the rate of methylation. However, unlike the situation with benzoic acid and to a lesser extent butyric acid, no inhibition of methylation by substrate concentrations up to 18 mM was observed.

Additionally, in contrast with carboxylic acid-methylation, no sharp fall in the rate of gaseous CH_3Cl release or attendant rapid increase in $C^2H_3^-$ incorporation into methylated product was displayed at supraoptimal concentrations of substrate, demonstrating that endogenous CH_3Cl biosynthesis is not significantly inhibited by higher concentrations of substrate (Fig. 2b).

The failure of phenol to exercise the tight regulation of the activity of both the methylating system and the rate of CH_3Cl biosynthesis that is shown by benzoic acid may possibly be ascribed to the fact that the maximum activity of the phenol-methylating system is associated with the idiophase, a stage where the precise control of the flux of metabolites characteristic of primary metabolism is not required. Alternatively, allosteric control of the system may be displayed only by the natural substrate, phenol, used in the assay.

Effect of exogenous C^2H_3Cl concentration on anisole biosynthesis and incorporation of $C^2H_3^-$

Washed mycelia were suspended in citrate buffer containing 1 mM-phenol with concentrations of C^2H_3Cl between 7.4 μM and 2.6 mM, and the rate of anisole formation together with the percentage $C^2H_3^-$ incorporated into anisole were determined (Fig. 3). Phenol methylation was markedly stimulated by increased C^2H_3Cl concentration. However, above approximately 100 μM , the methylating system appeared to be saturated with C^2H_3Cl . In this plateau region, a linear relationship ($r = 0.976$, $P < 0.001$) between the logarithm of % $C^2H_3^-$ incorporation and the logarithm of exogenous C^2H_3Cl concentration is apparent, the interdependence being defined by the equation

$$\log [\%C^2H_3^- \text{ incorp.}] = \log 4.55 + 0.25 \log [C^2H_3Cl \text{ concn.}]$$

This relationship is of the same form as that found with carboxyl methylation when the enzyme was saturated

Fig. 3. Effect of exogenous C^2H_3Cl concentration on anisole formation and incorporation of C^2H_3 - label into anisole in washed mycelia of *P. pomaceus*. Washed mycelia were incubated as described in Methods with 1 mM-phenol and concentrations of C^2H_3Cl between 7.5 μM and 2.4 mM. Anisole formation and incorporation of C^2H_3 - label into anisole were measured after 12 h. ○, Anisole production; ●, logarithm (base 10) of % anisole labelled with C^2H_3 .

with either benzoic or butyric acid as substrate, and conforms to the empirically derived Freundlich adsorption isotherm employed to predict adsorption from the gas phase or solution at a solid interface. McNally *et al.* (1990) interpreted this finding as suggesting that within the cell exogenous C^2H_3Cl competes with endogenously synthesized CH_3Cl at a membrane surface on opposite sides of which are located the CH_3Cl -biosynthesizing system and the CH_3Cl -utilizing system, each of them probably physically associated with the membrane. It would therefore appear that the phenol-methylating system, although biochemically distinct from the carboxyl-methylation system, is physically associated with the endogenous CH_3Cl -biosynthesizing enzyme in a manner very similar to that of the carboxyl-methylation system.

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