Appl Microbiol Biotechnol (1996) 45:744–749

ORIGINAL PAPER

D. Jahng · T. K. Wood

Metal ions and chloramphenicol inhibition of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b

Received: 10 June 1995/Received last revision: 7 March 1996/Accepted: 11 March 1996

Abstract Transcription of soluble methane monooxygenase (sMMO) of methanotrophs is tightly regulated by low concentrations of copper ions [Cu(II); e.g., transcription is completely repressed at copper concentrations higher than 0.86 μmol/g dry cell weight]. In addition to this genetic-level regulation, copper ions have been shown to inhibit the in vitro activity of sMMO from the type X methanotroph *Methylococcus capsulatus* (Bath) by inactivating only the reductase component of this enzyme (Green et al. 1985). In this study, in vitro sMMO inhibition by 12 metal ions and 10 medium ingredients was investigated for the first time using sMMO purified from the type II methanotroph *Methylosinus trichosporium* OB3b. Cu(I) and Cu(II) decreased sMMO activity of *Methylosinus trichosporium* OB3b by inhibiting not only the reductase but the hydroxylase component as well. Ni(II) also inhibited both enzyme components, but the inhibition was weaker than with copper ions. Zn(II) inhibited sMMO by lowering the activity of the hydroxylase only. Other transition metals such as Co(II), Mn(II), Fe(II) and Fe(III) did not show considerable impact on sMMO activity. The inhibition mechanisms were not determined, but Ni(II) and Zn(II) aggregated the reductase component of sMMO, and Zn(II) also precipitated the hydroxylase component. Cu(II) caused the reductase to precipitate, but Cu(I) did not aggregate either sMMO component. The aggregated proteins could not be dissolved in the solution of ethylenediaminetetraacetic acid disodium salt. Little or no sMMO inhibition was observed with various medium components examined including glucose, methanol, ethanol, dimethyl sulfone, ammonium chloride, methylamine (at a 500 molar ratio of medium component to the hydroxylase), kanamycin, and isopropylthiogalactopyranoside (at a molar ratio of 50); however, chloramphenicol inhibited sMMO at a molar ratio of 50.

D. Jahng · T. K. Wood (✉)
Department of Chemical and Biochemical Engineering,
University of California, Irvine, Irvine, CA 92717–2575, USA.
Fax: 714 824 2541
E-mail: tkwood@uci.edu

Introduction

Soluble methane monooxygenase (sMMO) is a multicomponent enzyme produced from type I [*Methylomonas methanica* 68–1 (Koh et al. 1993)], type II [*Methylosinus trichosporium* OB3b (Cardy et al. 1991) and *Methylocystis* strain M. (Nakajima et al. 1992)], and type X [*Methylococcus capsulatus* (Bath) (Pilkington and Dalton 1990)] methanotrophs. sMMO oxidizes methane to methanol, which is the first step of both the carbon-assimilation and respiration pathways in methanotrophs (Anthony 1982). This enzyme and methanotrophic microorganisms have received much attention for ecological (global-carbon cycle), commercial (e.g., single-cell-protein production from methane), and environmental (hazardous-waste treatment) reasons (Lidstrom 1990). Trichloroethylene degradation has been extensively studied (Brusseau et al. 1990; Oldenhuis et al. 1989, 1991; Tsien et al. 1989) because of the high rate of trichloroethylene oxidation and wide substrate range of sMMO (Fox et al. 1990a).

Soluble methane monooxygenase from *M. trichosporium* OB3b is composed of three catalytic proteins: the hydroxylase (which oxidizes methane or trichloroethylene), the reductase (which takes electrons from NADH), and component B (which is believed to regulate electron transfer from the reductase to the hydroxylase) (Fox et al. 1989). The hydroxylase (molecular mass 245 kDa) is a dimer of three different subunits, $(\alpha\beta\gamma)_2$ (Fox et al. 1989) and contains two dinuclear-iron centers per molecule at each active site (Fox et al. 1993). The reductase is a single protein (molecular mass 39.7 kDa) and contains one mole of flavin-adenine dinucleotide (FAD) and one mole of

[2Fe-2S] center per mole of the reductase (Fox et al. 1993). Component B is a monomeric protein with molecular mass 15.8 kDa and does not contain a prosthetic group or cofactor (Fox et al. 1993). Component B is not absolutely essential for sMMO activity but affects the efficiency of the reaction (Fox et al. 1989).

It is well known that sMMO expression in methanotrophs is repressed by copper ions [sMMO is expressed for concentrations lower than 0.86 μmol/g dry cell weight (Barta and Hanson 1993) or, generally, when the copper concentration is lower than 1 μM (Burrows et al. 1984)]. Above this concentration, the particulate (membrane-bound) form of methane monooxygenase (pMMO) is produced (Nguyen et al. 1994). To overcome this natural regulation, a *M. trichosporium* OB3b mutant has been obtained that expresses sMMO in the presence of copper probably because of a deficiency in copper transport (Phelps et al. 1992).

However, in vitro activity of sMMO in the presence of metals has not been studied extensively, and some results are controversial. Green et al. (1985) examined the effect of various metal ions on in vitro activity of purified sMMO from *M. capsulatus* (Bath) and found that the reductase component is inactivated irreversibly by copper ions (but the hydroxylase was not affected). In contrast, the sMMO from the soluble fraction of cell lysates of *M. trichosporium* OB3b is not as sensitive to copper ions as purified sMMO from *M. capsulatus* (Bath) (Fitch et al. 1993). Only a 23% decrease in activity of sMMO is observed in the presence of 15 μM Cu(II). In vitro studies using purified sMMO from *M. trichosporium* OB3b have not been conducted previously; therefore, this study on metal inhibition of whole sMMO and its components from *M. trichosporium* OB3b was performed.

In addition to metal ions, effects of commonly used medium components including glucose, ammonium ions (NH_4^+), isopropyl-β-D-1-thiogalactopyranoside (IPTG), and antibiotics (chloramphenicol and kanamycin) were also examined. These results can be utilized for optimizing the growth medium for maximum sMMO activity since glucose and ammonium ions are likely choices for a carbon and a nitrogen source in formulating cultivation media for recombinant strains expressing sMMO. Either the *tac* or *lac* promoter may also be adopted for transcriptional control of the *mmo* locus in which case IPTG is a common inducer (Jahng and Wood 1994). Antibiotics are also used to select cells harboring *mmo*-containing plasmids in the laboratory, so the effects of antibiotics on sMMO activity were discerned.

Materials and methods

Soluble methane monooxygenase assay

Purified soluble methane monooxygenase (sMMO) from *M. trichosporium* OB3b (ATCC 35070) was a generous gift from Professor John D. Lipscomb (University of Minnesota, Minneapolis, Minn.) and stored at $-85°C$. Purification procedures of sMMO from *M. trichosporium* OB3b are listed elsewhere (Fox et al. 1989). The hydroxylase, component B, and the reductase of sMMO were obtained in separate tubes, and their concentrations were measured in 25 mM MOPS (3-[N-morpholino]propanesulfonic acid, > 99.5%); Sigma, St. Louis, Mo., pH 7.5, from extinction coefficients of 504 $mM^{-1}cm^{-1}$ at 280 nm, 20.8 $mM^{-1}cm^{-1}$ at 282 nm, and 18.6 $mM^{-1}cm^{-1}$ at 458 nm respectively (Lipscomb personal communication).

The sMMO whole-enzyme assay (oxidation of propene to propene oxide) was performed using slight modifications of Fox et al. (1990b). The hydroxylase, component B, and the reductase were added at a 1:2:1 molar ratio (5–10 nmol:10–20 nmol:5–10 nmol) throughout this study. The concentration of NADH (USB, Cleveland, Ohio) in the assay solutions was 2 mM, and 6 ml gas-phase propene (at least 99%; Aldrich, Milwaukee, Wis.) was supplied as a substrate of sMMO. Propene oxide was assayed by using a gas chromatograph (GC) equipped with a flame-ionization detector (Hewlett Packard GC 5890 Series II, Wilmington, Del.) and a glass Porapak Q column (183 cm × 6.3 mm outer diameter, Hewlett Packard). Nitrogen was used as a carrier gas (60 ml/min). Temperatures of the injector, the column, and the detector were 225°C, 215°C, and 250°C respectively.

Hydroxylase assay

Hydroxylase activity was assayed using hydrogen peroxide as both an oxygen and an electron source so that NADH, oxygen, component B, and the reductase of sMMO were not required for the oxidation of propene. For this assay, saturation kinetics hold for hydrogen peroxide concentrations at least up to 300 mM for sMMO from *M. capsulatus* (Bath) (Jiang et al. 1993) and 350 mM for sMMO from *M. trichosporium* OB3b (Andersson et al. 1991). Hydrogen peroxide (100 mM, Fisher Scientific, Tustin, CA) was used throughout these experiments. All other conditions (including the amount of the hydroxylase) were identical to those used for the whole sMMO enzyme assay.

Reductase assay

Reductase activity was assayed by measuring the rate of decrease in absorbance at 600 nm resulting from the reduction of 2,6-dichloroindophenol (DCIP) (Fox et al. 1990b). Reductase (0.1–1.0 nmol) was added to a 1-ml spectrophotometer cuvette containing 150 μM DCIP (Sigma) in 50 mM MOPS, pH 7.0. The reaction was initiated by adding NADH (the final concentration in the solution was 0.5 mM) to the cuvette, and the temperature was maintained at 23°C. The rate of decrease in the absorbance at 600 nm (activity of the reductase) was measured for the first 10 s of the reaction. The specific activity of the reductase (μmol DCIP reduced min^{-1} mg $reductase^{-1}$) was calculated using the extinction coefficient of DCIP ($\varepsilon_{600} = 13\ mM^{-1}cm^{-1}$) (Fox et al. 1990a).

Metal ions and medium components

Metal ions (CsCl, LiCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H2O$, $FeCl_3$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, CuCl, $CuCl_2 \cdot 2H_2O$, and $CuSO_4 \cdot 5H_2O$ at a molar ratio of 0–100 mol ions/mol hydroxylase) and various medium components (glucose, methanol, ethanol, ammonium chloride, methylamine, and dimethylsulfone at a molar ratio of 500; IPTG, kanamycin, and chloramphenicol at a molar ratio of 50) were added to the enzyme assay solutions. All chemicals added to the enzyme assay solution were purchased from Fisher, except ethanol (Quantum, Tuscola, Ill.), methylamine (Aldrich), and cuprous chloride

(Sigma). IPTG was a dioxane-free grade. Chloramphenicol was dissolved in dimethylsulfone (Fisher; 400 mg/ml), and other compounds were dissolved in distilled water. All of the stock solutions prepared were immediately used to avoid possible oxidation of materials (e.g., oxidation of ferrous ions to ferric ions).

Determination of amount of aggregated reductase in the presence of copper ions

To determine the amount of aggregated reductase in the presence of Cu(II) ions, the concentration of unaggregated reductase in the soluble fraction was determined. After 5 min incubation at room temperature, 1.15 ml reductase solution (5 nmol reductase in 50 mM MOPS, pH 7.0) in the presence of various concentrations of cupric sulfate was microcentrifuged for 1 min at room temperature, and the absorbance at 458 nm of 1 ml supernatant ($\varepsilon_{458} = 18.6$ $mM^{-1}cm^{-1}$) (Lipscomb 1995) was measured to quantify the remaining amount of reductase in soluble form.

Results

Inhibition of whole soluble methane monooxygenase (sMMO) by metal ions

Among the 12 metal ions examined, Cu(I), Cu(II), Ni(II), and Zn(II) ions were found to inhibit whole sMMO enzyme activity (Table 1). Alkaline metals such as Mg(II) and Ca(II) did not show any inhibition, and some transition metals, including iron species (ferrous and ferric ions), Co(II), and Mn(II), were not inhibitory either at the same concentration (molar ratio of 25) as that of inhibitory metals. In fact, ferrous ions in a buffer for the purification of sMMO from *M. trichosporium* OB3b are known to reduce the loss of sMMO activity during a long purification procedure (Fox et al. 1989).

sMMO inhibition was not due to sulfate or chloride ions since calcium chloride and ferrous sulfate did not inhibit sMMO activity (Table 1). For all of the sMMO-inhibiting metal ions except Cu(I), sMMO aggregation was observed (sMMO-containing solutions turned turbid). This protein aggregation seemed to be irreversible since the turbidity of the solution with Cu(II), Ni(II), or Zn(II) did not disappear after addition of 250 μM EDTA · 2Na (ethylenediaminetetraacetic acid disodium salt).

Although these results were similar to metal-ion inhibition of sMMO from *M. capsulatus* (Bath) (Green et al. 1985), there are some notable differences in that Ni(II) inhibited sMMO from *M. trichosporium* OB3b but not sMMO from *M. capsulatus* (Bath), and the degree of inhibition by copper ions is more severe for sMMO from *M. capsulatus* (Bath) (complete loss of sMMO activity at a molar ratio, copper/reductase, of 40).

Inhibition of the hydroxylase by Cu(I), Cu(II), Ni(II), and Zn(II)

To discern which sMMO component (the hydroxylase or the reductase) was inhibited by Cu(I), Cu(II), Ni(II), and Zn(II), these four metal species were added individually to separate hydroxylase- and reductase-containing solutions. Hydrogen peroxide served both as electron and oxygen donor in the hydroxylase assay (without component B and the reductase of sMMO).

Except for weak reductase inhibition by Ni(II), all of the metal ions that inhibited whole sMMO enzyme decreased the activity of the hydroxylase (Fig. 1). The

Table 1 In vitro inhibition of whole soluble methane monooxygenase by various metal ions. The molar ratio of metal ions to the hydroxylase was 25, and 5 nmol hydroxylase, 10 nmol component B, and 5 nmol reductase were used. The specific activity, measured after 20 min of incubation, was 424 nmol propene oxide min^{-1} mg $hydroxylase^{-1}$

Metal ions	Relative activity	Protein aggregation
No metals	1.00	No
CsCl [Cs(I)]	0.98	No
LiCl [Li(I)]	0.90	No
$CaCl_2 \cdot 2H_2O$ [Ca(II]	1.00	No
$MgSO_4 \cdot 7H_2O$ [Mg(II)]	0.97	No
$MnSO_4 \cdot H_2O$ [Mn(II)]	1.00	No
$FeSO_4 \cdot 7H_2O$ [Fe(II)]	1.00	No
$FeCl_3$ [Fe(III)]	0.86	No
$CoCl_2 \cdot 6H_2O$ [Co(II)]	0.82	No
$NiCl_2 \cdot 6H_2O$ [(Ni(II)]	0.62	Yes
$ZnSO_4 \cdot 7H_2O$ [Zn(II)]	0.39	Yes
CuCl [Cu(I)]	0.39	No
$CuCl_2 \cdot 2H_2O$ [Cu(II)]	0.37	Yes
$CuSO_4 \cdot 5H_2O$ [Cu(II)]	0.34	Yes

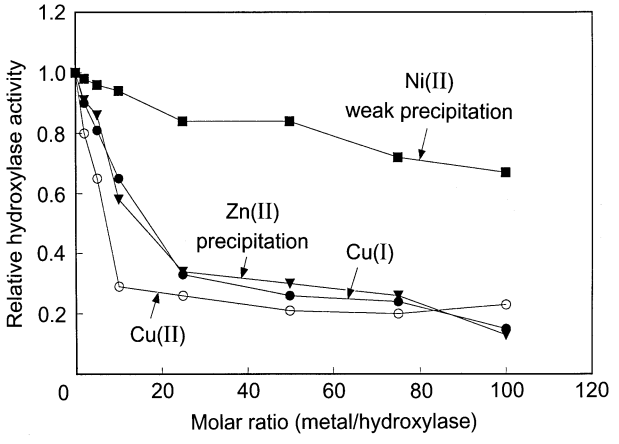

Fig. 1 Inhibition of the hydroxylase purified from *M. trichosporium* OB3b by Cu(I), Cu(II), Zn(II), and Ni(II) ions. Concentrations of the hydroxylase and hydrogen peroxide were 20 μM and 100 mM respectively. The specific activity of the hydroxylase without metal ions was 217 nmol propene oxide min^{-1} mg $hydroxylase^{-1}$

hydroxylase was aggregated by Zn(II), which resulted in a turbid solution. Addition of Ni(II) also gave rise to hydroxylase aggregation, but the turbidity of the solution was weaker than that with Zn(II). Hydroxylase aggregation by these two metal ions seemed to be irreversible since addition of EDTA · 2Na (2 mM) to the solution containing 1 mM metal ions (a metal/hydroxylase molar ratio of 50) did not re-solubilize the aggregated hydroxylase. Both Cu(I)- and Cu(II)-containing hydroxylase solutions did not show any sign of protein aggregation.

Inhibition of the reductase by Cu(I) and Cu(II)

Among the four metal ions inhibiting whole sMMO enzyme and the hydroxylase, Cu(I) and Cu(II) drastically lowered the reductase activity (22% and 20% of the activities respectively remained at a molar ratio of 10), but Ni(II) and Zn(II) did not show significant inhibition (only 16% of the activity was lost at a molar ratio of 50 for both metal ions) (Fig. 2). The degree of inhibition of the reductase by Cu(I) and Cu(II) was apparently similar; however, addition of Cu(II) to the reductase solution caused protein aggregation. Ni(II) also gave rise to very weak precipitation. Inhibition of reductase activity by Cu(II) seemed to be due to the aggregation of reductase since the soluble portions of the reductase decreased as Cu(II) was increased [at molar ratios Cu(II)/reductase of 0, 10, 25, and 50, the soluble fractions of the reductase were 0.99, 0.42, 0.39, and 0.28 respectively]. However, the soluble fractions were about twice as high as the remaining reductase activity at the same molar ratio of Cu(II) to the reductase [e.g., 20% of the reductase activity remained and 42% of the reductase was soluble with Cu(II) at a molar ratio of 10].

According to Green et al. (1985), the reductase of *M. capsulatus* (Bath) is also inhibited by Cu(I) and Cu(II).

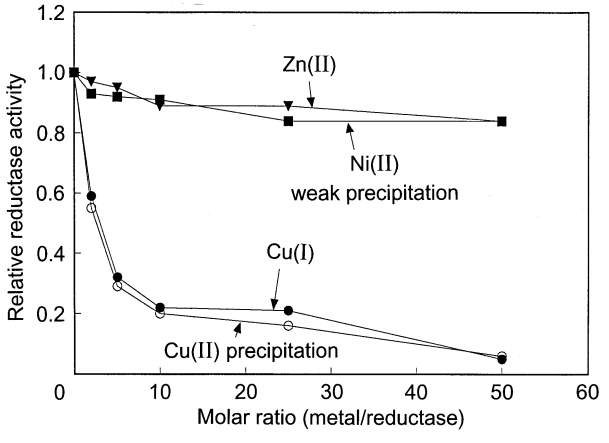

Fig. 2 Inhibition of the reductase by Cu(I) and Cu(II). The specific activity of the reductase without metal ions was 131 μmol dichloroindophenol min^{-1} mg $reductase^{-1}$

They found that four atoms of Cu(II) irreversibly bind to the reductase, disrupt its protein structure, and inhibit the [2Fe-2S] center and FAD; the reductase activity is not restored by adding excess FAD to the copper-treated reductase. This disruption of the reductase structure results in its solution changing color: the original dark-brown color turns to pale green upon the addition of Cu(II). The same color change was observed in this study for the Cu(II)-treated reductase solution of *M. trichosporium* OB3b. On the basis of these observations, it is suspected that Cu(II) disrupts the tertiary structure of the reductase from both methanotrophs, which results in the loss of the iron-sulfur center and FAD and aggregation of the insoluble, unfolded reductase.

Inhibition of sMMO by chloramphenicol

To investigate the effects of various common medium components (particularly, components used in recombinant-cell cultivation for sMMO expression), glucose, kanamycin, chloramphenicol, IPTG, dimethylsulfone and sMMO substrates (e.g., methanol, methylamine, ethanol, ammonium ions) were added to the sMMO assay solutions. Most of the additives did not cause significant loss of sMMO activity in the tested ranges of concentrations; however, ammonium chloride and methylamine inhibited whole sMMO activity by 10% (at a molar ratio of 500), and chloramphenicol was found to inhibit the sMMO enzyme by 35% (at a molar ratio of 50). Chloramphenicol is also known to inhibit the phenol hydroxylase (Shingler 1995a) of *Pseudomonas* strain CF600, which is similar to the hydroxylase component of sMMO in terms of the function (hydroxylation of substrates) (Powlowski and Shingler 1990) and contains the dinuclear-iron center as a prosthetic group (Shingler 1995b).

Discussion

The soluble methane monooxygenases from two different methanotrophs [*M. trichosporium* OB3b and *M. capsulatus* (Bath)] are closely related enzymes in terms of function (methane oxidation) and protein primary structures (Murrell 1992). The amino-acid sequences of both sMMO show more than 78% homology for all components (the α subunits of the two hydroxylases have 94% similarity). Even so, each sMMO behaved differently in the presence of metal ions. The in vitro activity of whole sMMO from *M. capsulatus* (Bath) is inhibited by Cu(I), Cu(II), Zn(II), Cd(II), and Ag(I), and these inhibitions are almost all due to loss of the reductase activity [copper does not inhibit the hydroxylase of *M. capsulatus* (Bath); Green et al. 1985]. However, for sMMO from *M. trichosporium* OB3b, Cu(I) and

Cu(II) inhibit *both* the reductase and the hydroxylase, and Zn(II) has a negative impact on the hydroxylase only. Ni(II) also slightly decreases the activities of the hydroxylase and reductase components from *M. trichosporium* OB3b, whereas sMMO from *M. capsulatus* (Bath) is not inhibited by Ni(II).

These differences in metal-ion inhibition are less surprising if the optimal temperatures of these two sMMO are taken into account. The optimal temperature for the sMMO from *M. trichosporium* OB3b is 30°C (Fox et al. 1990b) whereas that for sMMO from *M. capsulatus* (Bath) is 45°C (Pilkington et al. 1990); hence, the enzymes are not identical and operate at significantly different temperatures even though they have very similar protein sequences.

The degree of copper inhibition of sMMO in vitro (Table 1) is stronger than that seen in *M. trichosporium* OB3b cell lysates (Fitch et al. 1993). Assuming that 10% total cell protein is sMMO in methane-grown *M. trichosporium* OB3b, a similar loss of sMMO activity occurs at molar ratio of 2 for purified sMMO compared to molar ratio of 18 in cell lysates. This difference is likely due to the nonspecific binding of copper ions to cell proteins of the cell lysates, which results in fewer copper ions acting on sMMO. Interestingly, however, complete loss of activities either of the hydroxylase or of the reductase with metal ions is not observed with purified sMMO as seen with cell lysates at a molar ratio of 90.

It is well known that Cu(II) plays a key role in the regulation of sMMO expression for both methanotrophs, *M. trichosporium* OB3b (Burrows et al. 1984) and *M. capsulatus* (Bath) (Stanley et al. 1983). In the presence of high concentrations of copper ions, the transcription of the *mmo* locus is suppressed so that sMMO cannot be synthesized (Murrell 1992). This study shows that copper ions not only repress sMMO expression at the level of transcription but also inhibit sMMO enzyme activity. With copper inhibition of both transcription and enzyme activity, sMMO expression is tightly regulated by copper ions.

Since copper ions inhibited the hydroxylase of sMMO without protein aggregation, copper inhibition may be the result of removal of iron from the dinuclear-iron center by copper [loss of hydroxylase activity by Zn(II) or Ni(II) is likely due to the interruption of hydroxylase structures (quaternary or tertiary), which results in protein aggregation as observed]. According to Atta et al. (1992, 1993), the apo-enzymes of the hydroxylase from *M. capsulatus* (Bath) and the ribonucleotide reductase from *Escherichia coli* (which contains a dinuclear-iron center) are reconstituted with iron and manganese, so exchange of iron with copper may also be chemically possible in the hydroxylase of sMMO.

Although intracellular concentrations of medium ingredients will be much lower than the concentrations whole cells see in the growth medium, the inhibition results obtained in this study should be considered for recombinant sMMO expression since the in vitro inhibition of sMMO components is significant at a molar ratio as low as 2 [e.g., 45% of the reductase activity is lost with Cu(II) at this molar ratio]. Therefore, even in recombinants that lack the wild-type promoter, metal ions such as Cu(I), Cu(II), Ni(II), and Zn(II) need to be eliminated from the growth medium if possible (e.g., unless cell growth is retarded significantly), to avoid sMMO inhibition by these metal ions. In addition, chloramphenicol should be avoided for plasmid-selection pressure because this antibiotic was also inhibitory to sMMO.

Acknowledgements This work was supported by the U. S. Army Research Office (grant DAAL03-92-G-0398). We are grateful for the purified sMMO generously provided by Professor John D. Lipscomb of the University of Minnesota.

References

Andersson KK, Froland WA, Lee S, Lipscomb JD (1991) Dioxygen independent oxygenation of hydrocarbons by methane monooxygenase hydroxylase component. New J Chem 15:411–415

Anthony C (1982) The biochemistry of methylotrophs, Academic Press, New York

Atta M, Nordlund P, Åberg AHE, Fontecave M (1992) Substitution of manganese for iron in ribonucleotide reductase from *Escherichia coli*. J Biol Chem 267:20682–20688

Atta M, Fontecave M, Wilkins PC, Dalton H (1993) Abduction of iron(III) from the soluble methane monooxygenase hydroxylase and reconstitution of the binuclear site with iron and manganese. Eur J Biochem 217:217–223

Barta TM, Hanson RS (1993) Genetics of methane and methanol oxidation in Gram-negative methylotrophic bacteria. Antonie Van Leeuwenhoek 64:109–120

Brusseau GA, Tsien H-C, Hanson RS, Wackett LP (1990) Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1:19–29

Burrows KJ, Cornish A, Scott D, Higgins IJ (1984) Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b. J Gen Microbiol 130: 3327–3333

Cardy DLN, Laidler V, Salmond GPC, Murrell JC (1991) Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b. Mol Microbiol 5: 335–342

Fitch MW, Graham DW, Arnold RG, Agarwal SK, Phelps P, Speitel GE, Georgiou G (1993) Phenotypic characterization of copper-resistant mutants of *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 59:2771–2776

Fox BG, Froland WA, Dege JE, Lipscomb JD (1989) Methane monooxygenase from *Methylosinus trichosporium* OB3b. J Biol Chem 264:10023–10033

Fox BG, Borneman JG, Wackett LP, Lipscomb JD (1990a) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry 29:6419–6427

Fox BG, Froland WA, Jollie DR, Lipscomb JD (1990b) Methane monooxygenase from *Methylosinus trichosporium* OB3b. Methods Enzymol 188:191–202

Fox BG, Hendrich MP, Surerus KK, Andersson KK, Froland WA, Lipscomb JD, Munck E (1993) Mossbauer, EPR, and ENDOR studies of the hydroxylase and reductase components of methane

monooxygenase from *Methylosinus trichosporium* OB3b. J Am Chem Soc 115:3688–3701

Green J, Prior SD, Dalton H (1985) Copper ions as inhibitors of protein C of soluble methane monooxygenase of Methylococcus capsulatus (Bath). Eur J Biochem 153:137–144

Jahng D, Wood TK (1994) Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 60:2473–2482

Jiang Y, Wilkins PC, Dalton H (1993) Activation of the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath). Biochim Biophys Acta 1163:105–112

Koh S-C, Bowman JP, Sayler GS (1993) Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, *Methylomonas methanica* 68-1. Appl Environ Microbiol 59:960–967

Lidstrom ME, Stirling DI (1990) Methylotrophs: genetics and commercial applications. Annu Rev Microbiol 44:27–58

Murrell JC (1992) Genetics and molecular biology of methanotrophs. FEMS Microbiol Rev 88:233–248

Nakajima T, Uchiyama H, Yagi O, Nakahara T (1992) Novel metabolite of trichloroethylene in a methanotrophic bacterium, *Methylocystis* sp. M, and hypothetical degradation pathway. Biosci Biotechnol Biochem 56:486–489

Nguyen H-H, Shiemke AK, Jacobs SJ, Hales BJ, Lidstrom ME, Chan SI (1994) The nature of the copper ions in the membranes containing the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). J Biol Chem 269: 14995–15005

Oldenhuis R, Vink RLJM, Janssen DB, Witholt B (1989) Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl Environ Microbiol 55:2819–2826

Oldenhuis R, Oedzes JY, Waarde JJVD, Janssen DB (1991) Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl Environ Microbiol 57:7–14

Phelps PA, Agarwal SK, Speitel GE, Georgiou G (1992) *Methylosinus trichosporium* OB3b mutants having constitutive expression of soluble methane monooxygenase in the presence of high levels of copper. Appl Environ Microbiol 58:3701–3708

Pilkington SJ, Dalton H (1990) Soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). Methods Enzymol 188:181–189

Pilkington SJ, Salmond GPC, Murrell JC, Dalton H (1990) Identification of the gene encoding the regulatory protein B of soluble methane monooxygenase. FEMS Microbiol Lett 72: 345–348

Powlowski J, Shingler V (1990) In vitro analysis of polypeptide requirements of multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. J Bacteriol 172:6834–6840

Shingler V (1995) Check points in phenol degradation and its regulation. In: Fox CF, Stein GS, Burger MM (eds) Keystone Symposium on Environmental Biotechnology, March 17, Lake Tahoe, Calif. J Cellular Biochem (Suppl 21A)

Stanley SH, Prior SD, Leak DJ, Dalton H (1983) Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. Biotechnol Lett 5:487–492

Tsien HC, Brusseau GA, Hanson RS, Wackett LP (1989) Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 55:3155–3161