Alkene monooxygenase from *Mycobacterium*: a multicomponent enzyme

SYBE HARTMANS,* FRANS J. WEBER, DIANNE P. M. SOMHORST† and JAN A. M. DE BONT

Division of Industrial Microbiology, Department of Food Science, Agricultural University Wageningen, PO Box 8129, 6700 EV Wageningen, The Netherlands

(Received 24 April 1991; revised 16 July 1991; accepted 30 July 1991)

A NADH- or NADPH-dependent alkene monooxygenase (AMO) activity has been detected in cell-free extracts of the ethene-utilizing *Mycobacterium* E3 and *Mycobacterium aurum* L1. The activity was not linear with protein concentration in the assay suggesting AMO is a multicomponent enzyme. The inhibition pattern of AMO activity was very similar to the inhibition patterns published for the three-component soluble methane monooxygenases. Fractionation of crude extracts revealed that combination of two fractions was required to restore alkene monooxygenase activity. The first fraction was inhibited by acetylene, indicating it contained an oxygenase component. The second fraction contained reductase activity which was absent from non-induced cells. This reductase activity is probably the NADH-acceptor reductase of AMO.

Introduction

Ethene-utilizing mycobacteria have been studied mainly in relation to the possibility of using them to produce optically active epoxides (Habets-Crützen *et al.*, 1985; Weijers *et al.*, 1988; Hartmans *et al.*, 1989). Most studies have been done with whole cells and as yet little is known about the epoxidation reaction at the enzyme level. De Bont & Harder (1978) demonstrated that the initial step in ethene metabolism in *Mycobacterium* E20 was the oxidation of ethene to epoxyethane by alkene monooxygenase (AMO). Low activities of AMO were also detected in crude extracts of *Mycobacterium* E20 (de Bont *et al.*, 1979).

Oxidation of short-chain alkenes by methane monooxygenases also results in epoxide formation. Several methane monooxygenases have been studied in the past decade (Anthony, 1986). The most detailed studies have been done by H. Dalton and co-workers (see Anthony, 1986, for a review) with the soluble methane monooxygenase (MMO) of the type I methanotroph *Methylococcus capsulatus* (Bath). The MMO of *Methylococcus capsulatus* (Bath) has been resolved into three components that are all required for monooxygenase activity. Component A exhibits oxygenase activity in the pres-

ence of components C (reductase) and B (regulatory protein). In the absence of protein B, components A and C exhibit 'NADH oxidase' activity, reducing oxygen to water at the expense of NADH (Anthony, 1986). From the facultative methane-utilizing *Methylobacterium* sp. strain CRL-26 a similar soluble MMO has been purified which did not require a regulatory type B component (Patel & Savas, 1987). Recently, it was shown that *Methylosinus trichosporium* OB3b, a type II methanotroph, also contains a three-component soluble MMO, very similar to the *Methylococcus capsulatus* (Bath) enzyme (Fox & Lipscomb, 1988; Fox *et al.*, 1989). Oxidation of alkenes to the corresponding epoxides by *Pseudomonas oleovorans* is also catalysed by a three-component monooxygenase, although this enzyme has no similarity to the soluble methane monooxygenases (May, 1979).

The methane- and alkane-utilizing bacteria oxidize a wide range of hydrocarbons (Colby *et al.*, 1977; May, 1979; Stirling & Dalton, 1979; Higgins *et al.*, 1983; Witholt *et al.*, 1990) due to the broad substrate specificity of the monooxygenases present. The alkene-utilizing bacteria generally only oxidize alkenes to the corresponding epoxides (van Ginkel *et al.*, 1987). Based on this substrate specificity of AMO activity in whole cells the enzyme is expected to differ from reported monooxygenases.

This report describes the initial characterization of AMO from the ethene-utilizing *Mycobacterium* E3 (Habets-Crützen *et al.*, 1984).

† Present address: Agrotechnical Research Institute, PO Box 17, 6700 AA Wageningen, The Netherlands.

Abbreviations: AMO, alkene monooxygenase; MMO, methane monooxygenase.

Methods

Strains and cultivation. *Mycobacterium* E3 was isolated with ethene (Habets-Crützen *et al.*, 1984) and *Mycobacterium aurum* L1 with vinyl chloride as carbon source (Hartmans *et al.*, 1985). Production of biomass was by growth on ethene in a fed-batch manner in a 2 or 10 litre Applikon fermenter at pH 7 and 30 °C. Ethene was supplied continuously as a 2% (v/v) mixture in air at a rate of 0.2 v.v.m. About half of the fermentation liquid, containing approximately 3 g biomass (wet wt) l⁻¹, was harvested daily. After harvesting, the volume which had been withdrawn from the fermenter was replaced with new mineral salts medium. If biomass was not harvested daily, mineral medium was supplied continuously at a rate of 0.02 h⁻¹. The mineral salts medium contains, per litre of deionized water: 1.55 g K₂HPO₄, 0.85 g NaH₂PO₄ · 2H₂O, 2.0 g (NH₄)₂SO₄, 0.1 g MgCl₂ · 6H₂O, 10 mg EDTA, 2 mg ZnSO₄ · 7H₂O, 1 mg CaCl₂ · 2H₂O, 5 mg FeSO₄ · 7H₂O, 0.2 mg Na₂MoO₄ · 2H₂O, 0.2 mg CuSO₄ · 5H₂O, 0.4 mg CoCl₂ · 6H₂O and 1 mg MnCl₂ · 2H₂O. Maintenance of strains, culture harvesting and storage of harvested cells were as previously described (Hartmans & de Bont, 1986).

Preparation of cell extracts. Crude cell extracts were prepared as described previously (Hartmans & de Bont, 1986) except that 10% (v/v) glycerol (87%) was added before sonication. All chromatographic steps were done at 4 °C. Dialysis of crude extracts was done overnight at 4 °C against 200 vols 50 mM-potassium phosphate buffer, pH 7.3, with 8.7% glycerol (buffer A).

Fractionation on a DEAE-Sephacrose CL-6B column (25 × 2.5 cm). Elution was with a linear gradient of 0 to 1 M-NaCl in 1 litre of buffer A at a flow rate of 0.8 ml min⁻¹. Routinely about 0.5 g protein was applied to the column. The protein elution pattern was very reproducible, facilitating the localization of fractions X and Y. Concentration of the pooled fractions (fraction X eluted at a NaCl concentration of about 220 mM and fraction Y eluted at about 300 mM-NaCl) from the DEAE-Sephacrose column was done by ultrafiltration with an Amicon 8050 concentrator with Filtron membranes. The concentrated protein solution was subsequently diluted (1:10) with buffer A and once again concentrated to remove most of the NaCl.

Enzyme assays

All assays were done at 30 °C. Spectrophotometric assays were done on a Perkin-Elmer 550A spectrophotometer. Activities are expressed in nmol product formed min⁻¹ (mg protein)⁻¹.

Alkene monooxygenase. This was assayed by analysing epoxypropane formation. Assays were done in serum bottles (35 ml) sealed with a rubber septum. The reaction mixture contained 2 µmol NADH, cell extract and buffer A in a total volume of 1 ml. The serum bottles were incubated at 30 °C in a reciprocating water-bath. After 2 min in the water-bath the reaction was started by the addition of 1 ml propene via the rubber septum. Epoxypropane formation was determined by taking headspace samples every 2 to 3 min during a total incubation time of about 20 min. Epoxypropane formation was linear during this time period. Inhibitors were tested by adding them to the assay mixture (incubated at 30 °C) 5 min before the reaction was started by the addition of propene. Extracts used in determining the pH optimum of AMO were prepared in demineralized water containing 0.85% (w/v) NaCl and 8.7% glycerol. Immediately after preparation, the extract was diluted 2-fold with the appropriate buffer (0.1 M-potassium phosphate for pH 7.0 to 8.0, and 0.1 M-Bis-Tris for pH 5.7 to 7.0) and used to determine AMO activity.

NAD(P)H-acceptor reductase. These activities were assayed as described by Colby & Dalton (1979) using potassium ferricyanide as artificial acceptor in buffer A.

Component X. This was assayed either by adding acetylene-inactivated crude extract or fraction Y to the assay. For both assays it was verified that component X was the rate-limiting component in the total assay mix.

Component Y. This was assayed by adding fraction X to the assay in amounts sufficient to ensure that component Y was the rate-limiting component in the total assay mix.

Inactivation with acetylene. Inactivation of extracts was done by incubating extracts under the same conditions as were used for the AMO activity assay except that propene was omitted and 5% (v/v) acetylene was added to the gas phase. After incubation for 30 min at 30 °C the gas phase was flushed with air to remove all acetylene.

Slab gel electrophoresis. Flat polyacrylamide gels (140 × 80 × 3 mm) were prepared containing 7.7% (w/v) polyacrylamide and 2.7% (w/v) cross-linker in 100 mM-phosphate buffer, pH 7.3. The vertical slab gels were run under non-denaturing conditions immersed in sodium phosphate buffer (50 mM, pH 7.3) in a GE-2/4 gel electrophoresis apparatus from Pharmacia. Protein samples applied onto the gel contained 10% (w/v) sucrose and 0.02% bromophenol blue. Electrophoresis was started at 200 V until the proteins entered the gel (15 to 20 min) and was subsequently continued at 60 V until the dye reached the bottom of the gel. NADH-reductase activity staining was done immediately after electrophoresis by incubating the gel in 30 ml of buffer A with 1 mM-NADH and 0.6 mM-4-nitro blue tetrazolium chloride in the dark at 30 °C for 30 to 60 min.

Analytical methods. Protein was determined by the Bradford (1976) method using bovine serum albumin as standard. Epoxypropane was determined by analysing 200 µl headspace samples on a Packard 430 gas chromatograph fitted with a stainless steel Porapak Q column (100–120 mesh, 110 cm × 1/8 in. i.d.) and a flame-ionization detector. The oven temperature was 180 °C and the carrier gas N₂ at 20 ml min⁻¹.

Chemicals. Ethene, propene and carbon monoxide were from Hoek Loos (Schiedam, The Netherlands). Epoxypropane and glycerol (87%) were from Merck. Acetylene was prepared from calcium carbide (Aldrich) as described by Burris (1974). NADH, NADPH and 4-nitro blue tetrazolium chloride were from Boehringer. *o*-Phenanthroline was from Janssen and 8-hydroxyquinoline was from Sigma. DEAE-Sephacrose CL-6B was from Pharmacia and membrane filters with a nominal cutoff of 10 kDa (Omega NMWL 10K) were from Filtron Corporation.

Results and Discussion

Determination of AMO activity

AMO activity was assayed by determining epoxypropane formation from propene by headspace analysis. The rate of epoxypropane degradation by crude extracts is insignificant and using headspace analysis the formation of 2 nmol epoxypropane could be detected. Cell-free extracts were routinely prepared in the presence of glycerol as this significantly enhanced the stability of AMO activity. Extracts of ethene-grown, but not glucose-grown cells of *Mycobacterium* strains E3 and L1 contained AMO activity. In both strains AMO activity was strictly dependent on NAD(P)H and oxygen. Extracts of *Mycobacterium* E3 contained slightly higher specific AMO activities than those from strain L1 and

Fig. 1. Relationship between AMO activity and the amount of protein in the assay.

were consequently used in most experiments. The pH optimum for AMO activity was at pH 7.3 to 7.4, with activities of about 10% of the maximum at pH 6.0 and pH 8.0. Ultracentrifugation of crude extract at 150000 *g* for 90 min resulted in complete recovery of activity in the supernatant indicating that AMO is a soluble enzyme. Elution of crude extracts over a G-10 Sephadex column resulted in significant loss of AMO activity, which could not be restored by the addition of the salt peak. Dialysis by dilution and subsequent concentration using a membrane with a 10 kDa cutoff was, however, possible without significant loss of AMO activity.

AMO, as with other soluble monooxygenases capable of oxidizing short-chain alkenes (Colby & Dalton, 1976; Fox & Lipscomb, 1988), probably consists of more than one component since activity as a function of the protein concentration in the assay mix gave a non-linear relationship (Fig. 1). From Fig. 1 specific activities up to 1.1 nmol epoxypropane min⁻¹ (mg protein)⁻¹ can be calculated. This is significantly lower than the epoxypropane formation rate of 16 nmol min⁻¹ (mg protein)⁻¹ reported for whole cells of *Mycobacterium* E3 (Habets-Crützen *et al.*, 1984). Preparation of extracts without glycerol gave initial specific activities which were about twofold higher.

The specific activities of AMO are also very low when compared with the specific activities of about 80 nmol epoxypropane min⁻¹ (mg protein)⁻¹ reported for MMO from *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977) and *Methylosinus trichosporium* OB3b (Fox & Lipscomb, 1988).

Inhibitors of AMO activity

Using crude extracts several potential inhibitors of AMO activity were tested (Table 1). *o*-Phenanthroline,

Table 1. Inhibitors of alkene monooxygenase activity

Activity was determined by adding propene after 5 min pre-incubation at 30 °C with inhibitor

Inhibitor	Concn	Inhibition (%)
KCN	1 mM	0
	5 mM	33
	10 mM	>90
<i>o</i> -Phenanthroline	1 mM	23
	5 mM	100*
8-Hydroxyquinoline	2 mM	53
Thiourea	10 mM	0
Allylthiourea	10 mM	0
NaN ₃	10 mM	0
2,2'-Dipyridyl	10 mM	0
Salicylic acid	10 mM	0
EDTA	10 mM	0
Carbon monoxide	20% (v/v)	<5
Acetylene	5% (v/v)	>85

* Activity levelled off to zero during the assay.

8-hydroxyquinoline and potassium cyanide all significantly inhibited AMO activity. Other chelating agents tested did not result in significant inhibition, suggesting that any metal ion involved in alkene oxidation by AMO is well-shielded from attack by most metal-binding compounds. AMO activity, inhibited 40% with 2 mM-*o*-phenanthroline, was restored completely by the addition of 2 mM-Fe²⁺, but not by adding Cu²⁺, Zn²⁺, Mn²⁺ or Mg²⁺, indicating that iron plays an essential role in AMO activity.

Carbon monoxide did not inhibit AMO, indicating that a P450 type of cytochrome is not involved. The strong inhibitory effect observed with acetylene has also been reported for several other monooxygenases (Hyman & Wood, 1985; Hyman & Arp, 1988; Prior & Dalton, 1985). Inhibition by acetylene is dependent on the presence of both oxygen and NADH, and is a result of the transformation of acetylene by the monooxygenase to a reactive species which subsequently inactivates the active centre of the enzyme (Prior & Dalton, 1985).

The observed inhibition pattern for AMO of *Mycobacterium* E3 is very similar to the inhibition patterns reported for the soluble methane monooxygenases of *Methylococcus capsulatus* (Bath) (Stirling & Dalton, 1977) and *Methylosinus trichosporium* OB3b (Scott *et al.*, 1981). Further characterization of the oxygenase component of the soluble MMO of *M. capsulatus* (Bath) (component A) revealed the presence of an unusual non-haem iron centre (Green & Dalton, 1988). The iron in component A could be removed by dialysis against 8-hydroxyquinoline, resulting in an inactive protein. Reconstitution of the iron centre by incubating with iron-EDTA also resulted in reactivation of protein A (Green & Dalton, 1988).

Separation of AMO components

AMO in crude extracts prepared without glycerol lost 50% of its activity after 5 h at 4 °C. The crude enzyme preparation could be stabilized by adding glycerol. With 2.6, 4.4 and 8.7% glycerol added to crude extracts, AMO activity half-life at 4 °C was extended to 9, 18 and 40 h respectively.

In all subsequent experiments 8.7% glycerol was added to extracts and buffers. The addition of β -mercaptoethanol, PMSF, DTT, EDTA, NADH, FeSO_4 , $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ as well as storage under anaerobic conditions did not affect the stability of AMO activity.

Separation of the individual components of AMO was attempted on a DEAE-Sephacrose CL-6B column. Fractions pooled progressively to give 50 ml pools were concentrated to assay AMO activity. No activity could be detected in any of these concentrated 50 ml pools. Combination of all concentrated fractions, however, resulted in the recovery of some AMO activity. Apparently, the phenomena which resulted in the irreversible inactivation of AMO activity after elution over a G-10 Sephadex column played a less-significant role in DEAE column chromatography. The rate-limiting component of AMO activity was located by combining concentrated fractions from the DEAE-Sephacrose CL-6B column with crude extract and assaying for increased AMO activities. The rate-limiting component, designated component X, was located in the pooled fractions eluting between 545 and 615 ml (Fig. 2). These pooled fractions did not exhibit any AMO activity without addition of crude extract. Using crude extract which had been stored for some time at -20 °C the increase in AMO activity upon addition of the fraction containing component X was even more significant: the activity of thawed crude extract containing 4.5 mg protein increased from 0.8 nmol epoxypropane min^{-1} to 10 nmol min^{-1} when 7.2 mg protein of fraction X was added.

A preparation containing this presumably most labile component X of AMO was used to locate a second fraction which upon combination with fraction X gave AMO activity. This second fraction was designated fraction Y.

At this stage it was verified that both components X and Y were present only in cells grown with ethene as growth substrate. Combination of fractions X (2.2 mg protein) or Y (1.2 mg protein) with crude extracts (7.8 mg protein) of glucose-grown cells did not result in detectable levels of AMO activity, indicating that components X and Y were indeed not present in extracts of glucose-grown cells. Furthermore, fractions containing components X and Y were also prepared from extracts of ethene-grown *Mycobacterium aurum* L1 cells. All combi-

nations of fractions X and Y of the two strains resulted in activity, indicating a certain degree of similarity between the AMOs of the two strains.

Characterization of fractions X and Y

In an initial characterization of fractions X and Y it was determined which of these fractions contained NADH-acceptor reductase activity by following the reduction of the artificial acceptor potassium ferricyanide (Colby & Dalton, 1979). Significant reductase activity was present in fraction Y. In a subsequent separation of fractions X and Y this assay was used to locate the reductase component Y more precisely (Fig. 2). Several peaks exhibiting reductase activity were located. Fractions from the peak with the highest reductase activity (670 to 730 ml) gave AMO activity upon combination with fraction X obtained by concentrating the protein peak which eluted between 545 and 615 ml. The location of fractions X and Y used in the activity determinations shown in Table 2 are depicted by the horizontal lines in Fig. 2.

As component X was assumed to be rate-limiting in crude extracts, the activity of crude extract can be assumed to be determined by the amount of component X present. The specific activity of fraction X in the presence of excess component Y was 0.42 nmol min^{-1} (mg protein) $^{-1}$, which is only slightly higher than the specific activity of crude extract [0.25 nmol min^{-1} (mg protein) $^{-1}$]. The apparent purification of the reductase component Y appeared to be slightly higher, with the specific activity increasing from 0.41 to 1.99 nmol min^{-1} (mg protein) $^{-1}$ (Table 2). These low increases in specific activities could indicate that a third component is required for AMO activity, as has been established for the soluble MMOs (Anthony, 1986; Fox *et al.*, 1989). If such a third component is required it is apparently present in limiting amounts in either fraction X or Y (or both). Attempts to further purify components X and Y should resolve this matter in the future.

Addition of fraction X to crude extracts which had been inactivated by incubation with acetylene restored AMO activity. As can be seen in Table 2 this gave the same specific activity [0.45 nmol min^{-1} (mg protein) $^{-1}$], based on fraction X protein, as the assay with excess component Y [0.46 nmol min^{-1} (mg protein) $^{-1}$]. Addition of fraction Y to acetylene-inactivated extracts did not result in restoration of AMO activity. Apparently, inactivation with acetylene results in inactivation only of component X. Inactivation of component X with acetylene is accomplished only in the presence of active AMO. Incubation of fractions X and Y separately with acetylene in the presence of NADH and oxygen for 30 min and subsequent removal of acetylene before combin-

Fig. 2. DEAE-Sepharose CL-6B fractionation of crude extract. Fractions of vol. 8 ml were collected. The horizontal lines indicate the elution volumes used to prepare fractions X and Y. NAD(P)H-acceptor reductase activities (●) were assayed with potassium ferricyanide as artificial acceptor.

Table 2. *Specific activities of alkene monooxygenase fractions*

cfe, cell-free extract (stored at 4 °C during preparation of X and Y); cfe/Ac, cell-free extract inactivated with acetylene; X and Y, preparations fractionated on the DEAE column as depicted in Fig. 2.

Protein in assay (mg)				Activity (nmol min ⁻¹)	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	
cfe	cfe/Ac	X	Y		X	Y
12.5	—	—	—	3.1	0.25	—
—	—	1.1	1.9	0.46	0.42	—
12.5	—	6.0	—	5.0	—	0.41
—	—	7.6	0.62	1.23	—	1.99
—	9.0	1.1	—	0.45	0.41	—

ing both fractions gave active AMO. Combination of fractions X and Y before incubation with acetylene resulted in inactivation of AMO activity. These results indicated that both preparations are essential to produce the AMO-inactivating product of acetylene and that fraction X appears to contain the AMO component which can be inactivated by acetylene. Prior & Dalton (1985) demonstrated that only one protein was labelled in soluble and particulate fractions of crude extracts of *Methylococcus capsulatus* (Bath) after incubation with [¹⁴C]acetylene. For the soluble MMO preparation the labelled polypeptide corresponded to the α -subunit of component A, the oxygenase component of the soluble MMO. By analogy with the soluble MMO of *Methylococcus capsulatus* (Bath), fraction X could very well contain the oxygenase component of AMO.

The specific activity of fraction Y when assayed with potassium ferricyanide was 16.6 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ compared to 230 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ reported for the purified component C of *Methylococcus*

Fig. 3. Non-denaturing gel electrophoresis showing NADH reductase activity stained with 4-nitro blue tetrazolium chloride. Lane A, crude extract of ethene-grown cells; lane B, crude extract of glucose-grown cells; lane C, first peak with reductase activity from DEAE column (at 530 ml); lane D, fraction X; lane E, fraction Y.

capsulatus (Bath) (Colby & Dalton, 1979). Using gel electrophoresis under non-denaturing conditions fraction Y was compared with several other samples after activity staining with 4-nitro blue tetrazolium chloride.

As can be seen in Fig. 3 a band with reductase activity at the same migration distance as the major reductase component of fraction Y was present only in crude extracts of ethene-grown cells. Fraction X and extracts of glucose-grown cells did not exhibit any detectable reductase activity at this migration distance. This indicates that the major band in the lane with fraction Y is probably the reductase component of AMO.

It is therefore concluded that in both of the mycobacteria studied a multicomponent AMO is induced after growth on ethene. In extracts of *Mycobacterium* E3 two fractions (designated X and Y) were located after separation on an anionic exchanger; upon combination these two fractions gave AMO activity. Based on inhibition experiments with acetylene it was concluded that fraction X contains an oxygenase component. Fraction Y contained significant reductase activity which is induced after growth with ethene. From the present data it is not clear if, in analogy with the soluble MMO, a third component is required for AMO activity. Attempts to further purify components X and Y should resolve this matter in the future.

References

- ANTHONY, C. (1986). Bacterial oxidation of methane and methanol. *Advances in Microbial Physiology* **27**, 113–210.
- DE BONT, J. A. M. & HARDER, W. (1978). Metabolism of ethylene by *Mycobacterium* E20. *FEMS Microbiology Letters* **3**, 89–93.
- DE BONT, J. A. M., ATTWOOD, M. M., PRIMROSE, S. B. & HARDER, W. (1979). Epoxidation of short chain alkenes in *Mycobacterium* E20: the involvement of a specific mono-oxygenase. *FEMS Microbiology Letters* **6**, 183–188.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- BURRIS, R. H. (1974). Methodology. In *The Biology of Nitrogen Fixation*, p. 23. Edited by A. Quispel. Amsterdam: North Holland Publishing Co.
- COLBY, J. & DALTON, H. (1976). The soluble methane mono-oxygenase from *Methylococcus capsulatus* strain (Bath). *Biochemical Journal* **157**, 495–497.
- COLBY, J. & DALTON, H. (1979). Characterization of the second prosthetic group of the flavoenzyme NADH-acceptor reductase (component C) of the methane mono-oxygenase from *Methylococcus capsulatus* (Bath). *Biochemical Journal* **177**, 903–908.
- COLBY, J., STIRLING, D. I. & DALTON, H. (1977). The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). *Biochemical Journal* **165**, 395–402.
- FOX, B. G. & LIPSCOMB, J. D. (1988). Purification of a high specific activity methane monooxygenase hydrolase component from a type II methanotroph. *Biochemical and Biophysical Research Communications* **154**, 165–170.
- FOX, B. G., FROLAND, W. A., DEGE, J. E. & LIPSCOMB, J. D. (1989). Methane monooxygenase from *Methylosinus trichosporium* OB3b. *Journal of Biological Chemistry* **264**, 10023–10033.
- VAN GINKEL, C. G., WELTEN, H. G. J. & DE BONT, J. A. M. (1987). Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. *Applied and Environmental Microbiology* **53**, 2903–2907.
- GREEN, J. & DALTON, H. (1988). The biosynthesis and assembly of protein A of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). *Journal of Biological Chemistry* **263**, 17561–17565.
- HABETS-CRÜTZEN, A. Q. H., BRINK, L. E. S., VAN GINKEL, C. G., DE BONT, J. A. M. & TRAMPER, J. (1984). Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. *Applied Microbiology and Biotechnology* **20**, 245–250.
- HABETS-CRÜTZEN, A. Q. H., CARLIER, S. J. N., DE BONT, J. A. M., WISTUBA, D., SCHURIG, V., HARTMANS, S. & TRAMPER, J. (1985). Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. *Enzyme and Microbial Technology* **7**, 17–21.
- HARTMANS, S., DE BONT, J. A. M., TRAMPER, J. & LUYBEN, K. CH. A. M. (1985). Bacterial degradation of vinyl chloride. *Biotechnology Letters* **7**, 383–388.
- HARTMANS, S. & DE BONT, J. A. M. (1986). Acetol monooxygenase from *Mycobacterium* Pyl cleaves acetol into acetate and formaldehyde. *FEMS Microbiology Letters* **36**, 155–158.
- HARTMANS, S., DE BONT, J. A. M. & HARDER, W. (1989). Microbial metabolism of short-chain unsaturated hydrocarbons. *FEMS Microbiology Reviews* **63**, 235–264.
- HIGGINS, I. J., BEST, D. J. & HAMMOND, R. C. (1983). New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. *Nature, London* **286**, 561–564.
- HYMAN, M. R. & ARP, D. J. (1988). Acetylene inhibition of metalloenzymes. *Analytical Biochemistry* **173**, 207–220.
- HYMAN, M. R. & WOOD, P. M. (1985). Suicidal inactivation and labelling of ammonia mono-oxygenase by acetylene. *Biochemical Journal* **227**, 719–725.
- MAY, S. W. (1979). Enzymatic epoxidation reactions. *Enzyme and Microbial Technology* **1**, 15–22.
- PATEL, R. N. & SAVAS, J. C. (1987). Purification and properties of the hydroxylase component of methane monooxygenase. *Journal of Bacteriology* **169**, 2313–2317.
- PRIOR, S. D. & DALTON, H. (1985). Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). *FEMS Microbiology Letters* **29**, 105–109.
- SCOTT, D., BRANNAN, J. & HIGGINS, I. J. (1981). The effect of growth conditions on intracytoplasmic membranes and methane monooxygenase activities in *Methylosinus trichosporium* OB3b. *Journal of General Microbiology* **125**, 63–72.
- STIRLING, D. I. & DALTON, H. (1977). Effect of metal-binding agents and other compounds on methane oxidation by two strains of *Methylococcus capsulatus*. *Archives of Microbiology* **114**, 71–76.
- WEIJERS, C. A. G. M., VAN GINKEL, C. G. & DE BONT, J. A. M. (1988). Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane- and alkene-utilizing bacteria. *Enzyme and Microbial Technology* **10**, 214–218.
- WITHOLT, B., DE SMET, M.-J., KINGMA, J., VAN BEILEN, J. B., KOK, M., LAGEVEEN, R. G. & EGGINK, G. (1990). Bioconversion of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors: background and economic potential. *Trends in Biotechnology* **8**, 46–52.