Butane monooxygenase of '*Pseudomonas butanovora*': purification and biochemical characterization of a terminal-alkane hydroxylating diiron monooxygenase

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Butane monooxygenase (sBMO) has been purified to homogeneity from the Gram-negative β -proteobacterium '*Pseudomonas butanovora*' and confirmed to be a three-component diiron monooxygenase system. The reconstituted enzyme complex oxidized C₃–C₆ linear and branched aliphatic alkanes, which are growth substrates for '*P. butanovora*'. The sBMO complex was composed of an iron-containing hydroxylase (BMOH), a flavo-iron sulfur-containing NADH-oxidoreductase (BMOR) and a small regulatory component protein (BMOB). The physical characteristics of sBMO were remarkably similar to the sMMO family of soluble multicomponent diiron monooxygenases. However, the catalytic properties of sBMO were quantitatively different in regard to inactivation in the presence of substrate and product distribution. BMOH was capable of ethene oxidation when supplied with H₂O₂ and ethene (known as the peroxide shunt), but this activity was at least three orders of magnitude less than that observed for the hydroxylase of sMMO of *Methylosinus trichosporium* OB3b. BMOH and BMOR were efficient in the oxidation of ethene in the absence of BMOB with regard to rate of reaction and product yield. Regiospecificity of sBMO was strongly biased towards primary hydroxylation, with $\geq 80\%$ of the hydroxylations occurring at the terminal carbon atom.

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INTRODUCTION

The utilization of aliphatic alkanes for carbon and energy is carried out by a diverse group of aerobic prokaryotes. Insertion of an oxygen atom into alkanes is catalysed by several unrelated enzymes, resulting in terminal and subterminal alcohols. Downstream metabolism of these products occurs by both terminal and subterminal pathways (Arp, 1999; Ashraf *et al.*, 1994). The best characterized of these enzymes are soluble methane monooxygenase (sMMO) (Kopp & Lippard, 2002; Lipscomb, 1994; Wallar & Lipscomb, 1996) and the integral membrane monooxygenase, AlkB (Shanklin *et al.*, 1997; van Beilen *et al.*, 2005). Regiospecificity is not an issue for methane as it is the only physiologically relevant substrate of sMMO. However, AlkB is highly regiospecific for hydroxylation at the

terminal carbon atom of C₅–C₁₆ alkanes. Recently, a family of cytoplasmic cytochrome P450 enzymes (the CYP153 family) has been identified and a few members from *Mycobacterium* sp. HXN-1500 and *Alcanivorax borkumensis* characterized (Funhoff *et al.*, 2006; van Beilen *et al.*, 2006). Studies of these enzyme systems constitute the bulk of the knowledge of the first step in alkane metabolism in prokaryotes.

Growth on C₂–C₄ gaseous alkanes is largely attributed to the *Corynebacterium-Nocardia-Mycobacterium-Rhodococcus* complex of Gram-positive bacteria (Ashraf *et al.*, 1994; McLee *et al.*, 1972; Perry, 1980). An exception to this association is the Gram-negative β -proteobacterium '*Pseudomonas butanovora*' (ATCC 43655). Although originally identified as a pseudomonad, 16S rDNA analysis of '*P. butanovora*' has shown that phylogenetically it is a member of the *Rhodocyclus* group, with the closest relatives belonging to the genus *Thauera* (Anzai *et al.*, 2000). '*P. butanovora*' was isolated from activated sludge sampled from an oil refining plant and was capable of aerobic growth on the linear alkanes C₂–C₉ (Takahashi *et al.*, 1980). The same enzyme system was used to oxidize both the gaseous and liquid alkanes (Hamamura *et al.*, 1999; Sluis *et al.*, 2002). The downstream metabolism, in the case

Abbreviations: BMOB, butane monooxygenase regulatory component; BMOH, butane monooxygenase hydroxylase; BMOR, butane monooxygenase reductase; MMOB, methane monooxygenase regulatory component; MMOH, methane monooxygenase hydroxylase; MMOR, methane monooxygenase reductase; sBMO, soluble butane monooxygenase; sMMO, soluble methane monooxygenase; T2MO, toluene 2-monooxygenase.

A supplementary table summarizing the purification of sBMO components is available with the online version of this paper.

of butane, was determined to be via the terminal alcohol 1-butanol, with subsequent oxidations leading to butyraldehyde and butyrate. Presumably butyrate was shunted to the tricarboxylic acid cycle via the β -oxidation pathway (Arp, 1999). Previous work, through partial enzyme purification and insertional mutagenesis, established the genetic identity of a soluble three-component diiron monooxygenase, termed soluble butane monooxygenase (sBMO) and confirmed the role of sBMO in alkane oxidation (Sluis *et al.*, 2002). That study also demonstrated the similarities of the *bmo* operon structure and the deduced amino acid sequence identities of the *bmo* genes to sMMO operons and gene products of the methanotrophs *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath. Several detailed *in vivo* studies have focused on cometabolism of environmentally important pollutants by '*P. butanovora*' for the purpose of bioremediation (Doughty *et al.*, 2005; Halsey *et al.*, 2005; Hamamura *et al.*, 1997), the regulation of the *bmo* operon (Doughty *et al.*, 2006; Sayavedra-Soto *et al.*, 2001, 2005) and altering the regiospecificity of sBMO by site-directed mutagenesis of the hydroxylase α -subunit (Halsey *et al.*, 2006).

This study presents the first report of the purification of all the components of a soluble three-component diiron monooxygenase, with physiologically relevant activity, that was responsible for the oxidation of physiologically relevant C_2 – C_9 alkanes as sources of carbon and energy. Physical characteristics and the roles for each of the subunits in catalysis are described. Product distribution studies revealed the strong bias that sBMO exhibits for terminal hydroxylation of C_3 – C_6 linear and branched alkanes. Comparisons were made between the properties of sBMO and those of other soluble multicomponent diiron monooxygenases, and the implication of these findings for the catalytic mechanism of sBMO are discussed.

METHODS

Chemicals. Gaseous alkanes were of reagent grade. Methane and ethene were purchased from Airco. Ethane was purchased from Matheson. Propane and butane were purchased from Airgas. Ethene oxide was purchased from Aldrich. Acetylene was produced from CaC_2 by the addition of H_2O . DEAE Sepharose FF, Sephacryl S300 HR and Superdex 75 FF were purchased from GE Healthcare. NADH was purchased from Research Organics and residual ethanol was removed by repeated freeze-drying from 25 mM PIPES pH 7.2. Bovine liver catalase was purchased from Sigma. Molecular mass standards were purchased from Bio-Rad. All other chemicals were obtained from Aldrich.

Bacterial cultivation. '*P. butanovora*' (ATCC 43655) was cultivated at 30 °C in liquid medium consisting of 36 mM KH_2PO_4 , 14 mM K_2HPO_4 , 2 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2 \cdot 2H_2O$, 30 mM KNO_3 , 2 μ M $Na_2MoO_4 \cdot 2H_2O$, 1 ml trace element solution (Wiegant & deBont, 1980), 10 ml vitamin solution (Lidstrom, 1988) and 500 μ M $FeSO_4 \cdot 7H_2O$ at pH 7.2. Butane was supplied as an overpressure 7% (v/v) in the headspace. Batch cultures were grown in a sealed 20 l glass carboy at 30 °C. Medium (15 l) was inoculated with 2 l overnight culture. The culture was constantly stirred and sparged with butane added as an overpressure to 7–10% (v/v) via a vacuum-pressure pump

with a 125 l Nalgene carboy serving as headspace. Cells were harvested (OD_{600} 1.0–1.5) by centrifugation (7000 g for 30 min at 10 °C), suspended in 25 mM PIPES (pH 7.2), and centrifuged again. The cell paste was frozen in liquid N_2 and stored at –70 °C. A yield of 2–3 g cells (1 culture)^{–1} was typical.

Preparation of cell-free extract. Approximately 60 g '*P. butanovora*' cell paste was suspended 1 : 1 (w/v) in 25 mM PIPES (pH 7.2), 1 mM DTT, 5% (v/v) glycerol and 200 μ M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (buffer A). The cells were disrupted by sonication by the method of Fox *et al.* (1990). The sonicated suspension was centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was removed by careful decanting, diluted to 200 ml with buffer A and centrifuged at 150 000 g for 2 h at 4 °C. The supernatant was decanted, adjusted to pH 7.2 and designated the cell-free extract.

Separation of soluble butane monooxygenase components.

Unless otherwise noted, the following purification steps for each of the components were carried out at 4 °C. '*P. butanovora*' cell-free extract was loaded onto a DEAE-Sepharose FF column (5.2 $\times$ 15 cm) equilibrated with buffer A at a linear flow rate of 28 cm h^{–1}. The column was washed with two column volumes of buffer A, followed by a 1.2 l gradient of 0–0.4 M KCl in buffer A at a linear flow rate of 14 cm h^{–1}. Fractions containing the monooxygenase components eluted in the following order and KCl concentrations, BMOH and BMOB (0.23–0.27 M) and BMOR (0.34–0.39 M).

Purification of BMOH. Fractions containing BMOH and BMOB were pooled, brought to 25% saturation with solid $(NH_4)_2SO_4$ and stirred at 20 °C for 1 h. The solution was centrifuged at 10 000 g for 30 min and the supernatant decanted. Solid $(NH_4)_2SO_4$ was added to the supernatant to 75% saturation and stirred as above. After centrifugation at 10 000 g for 30 min, the pellet was suspended in a minimal volume of 25 mM PIPES (pH 7.2), 1 mM DTT, 5% (v/v) glycerol, 100 μ M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1 M KCl (buffer B). This fractionated solution was loaded onto a Sephacryl S-300 HR column (3.2 $\times$ 90 cm) equilibrated with buffer B at a linear flow rate of 15 cm h^{–1}. Fractions containing BMOH were pooled, concentrated and dialysed in 25 mM PIPES (pH 7.2), 1 mM DTT, 5% (v/v) glycerol and 100 μ M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ via ultrafiltration, frozen in liquid N_2 and stored at –70 °C.

Purification of BMOB. Pooled fractions containing BMOB from the Sephacryl S-300 HR column were concentrated and dialysed via ultrafiltration in 25 mM PIPES (pH 7.2) and 0.2 M KCl. The concentrated protein solution was applied to a Q Sepharose FF column (2.2 $\times$ 11 cm) equilibrated in the same buffer at a linear flow rate of 30 cm h^{–1}. The small component was eluted with a 0.2 l gradient from 0.2 to 0.35 M KCl at a linear flow rate of 15 cm h^{–1}. Fractions containing BMOB were pooled, concentrated and dialysed in 25 mM PIPES (pH 7.2) and 0.15 M KCl via ultrafiltration. This solution was applied to a Sephadex 75 FF column (1.6 $\times$ 25 cm) equilibrated in the same buffer at a linear flow rate of 15 cm h^{–1}. BMOB fractions were pooled, concentrated and dialysed via ultrafiltration in 25 mM PIPES (pH 7.2), frozen in liquid N_2 and stored at –70 °C.

Purification of BMOR. BMOR fractions were pooled and diluted with two volumes of 25 mM PIPES (pH 7.2) and 5 mM sodium thioglycollate. This solution was applied to a Q Sepharose FF column (2.2 $\times$ 11 cm) equilibrated in the above buffer at a linear flow rate of 30 cm h^{–1}. BMOR was eluted from the column with a 0.2 l gradient from 0.25–0.4 M KCl at a linear flow rate of 15 cm h^{–1}. Fractions containing BMOR were pooled, concentrated via ultrafiltration and applied to a Sephadex 75 FF column (1.6 $\times$ 25 cm). The column was equilibrated in 25 mM PIPES (pH 7.2), 0.15 M KCl and 5 mM sodium thioglycollate and developed at a linear flow rate of 15 cm h^{–1}.

Fractions containing reductase were pooled, concentrated and dialysed via ultrafiltration in 25 mM PIPES (pH 7.2) containing 5 mM sodium thioglycollate, frozen in liquid N₂ and stored at -70°C .

NADH-coupled enzyme assays. Soluble butane monooxygenase activity was assayed by following the epoxidation of ethene to ethene oxide by gas chromatography (Hamamura *et al.*, 1997). BMOH (1 μM), BMOB (0.1–4 μM) and BMOR (0.2–8 μM) were placed in 25 mM PIPES (pH 7.2) in a total volume of 1 ml in a 10 ml reaction vial and sealed with a butyl rubber septum. Catalase, when present, was added to 4000 units per reaction. BMOR, when necessary, was added to 10 or 100 mM H₂O₂ in the above buffer and incubated at 30°C for 30 min with shaking prior to addition of catalase and other components. Ethene (2 ml) was added as an overpressure to the headspace and allowed to equilibrate at 30°C for 5 min. The reaction was started by the addition of NADH to 5 mM. Ethene oxide formation was analysed from 0.1 ml reaction vial headspace. Reactions to assess alcohol production contained optimized concentrations of monooxygenase components in 0.5 ml total volume. Gaseous alkanes were added as an overpressure to the headspace (3 ml). Liquid alkanes were added as neat liquid (5–10 μl). After addition of alkane the reaction was placed at 30°C for 5 min and initiated by addition of NADH to 5 mM. The reaction was allowed to proceed for 5 min. The formation of alcohols was analysed by gas chromatography on a 0.2×180 cm 0.2% Carbowax 1500 on Graphpac GC 80/100 column by injection of 1 μl of the liquid phase.

H₂O₂-coupled enzyme assays. BMOH (200 μM) was placed in a 10 ml reaction vial in 25 mM PIPES, pH 7.2, and sealed with a butyl rubber septum. The headspace was subjected to evacuation and replacement with N₂ for 3 cycles. Ethene (3 ml) was added to the headspace as an overpressure and allowed to equilibrate at 30°C for 5 min. The reaction was initiated by the addition of H₂O₂. Ethene oxide was analysed by GC as described above and H₂O₂ was measured by the method of Zhang & Lipscomb (2006). Assays of recoverable activity were carried out as described above for NADH-coupled ethene oxidation with optimal concentration of BMOB and BMOR in the presence of catalase.

Other methods. Denaturing SDS-PAGE was performed as described by Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976) as modified by Nelson *et al.* (1982). Optical spectra were recorded with a Beckman DU 640 spectrophotometer. Sedimentation equilibrium experiments were carried out with a Beckman Optima XL-A analytical ultracentrifuge equipped with a Beckman An-60 Ti rotor. The reference and sample cell assemblies were monitored at a wavelength of 280 nm with a rotor speed of 22 000 r.p.m. at 20°C . N-terminal amino acid sequence analysis was performed by Edman degradation with an Applied Biosystems 492 Procise Protein Sequencer coupled with a model 140C analyser by the Protein Facility at Iowa State University. Iron content was determined spectrophotometrically by the method of Percival (1991). H₂O₂ reduction was assayed with a Clark-style electrode measuring the evolution of oxygen as previously described (Arp, 1999).

RESULTS

Purification of butane monooxygenase components

The addition of 500 μM FeSO₄ to culture medium was found to increase *in vivo* sBMO activity by approximately threefold and resulted in the purification of high-activity preparations of BMOH. Cell-free extracts exhibited

reproducible butane monooxygenase activity. It should be noted that there were differences in activity from preparation to preparation, ranging from 200 to 600 nmol min⁻¹ (mg hydroxylase)⁻¹, that were most likely due to culture conditions and harvesting technique. The activity was fully contained in the soluble cell-free extract. Individual components were screened for restoration of activity as previously described (Sluis *et al.*, 2002). Purified sBMO components are shown by denaturing SDS-PAGE in Fig. 1 and the UV-visible spectra are shown in Fig. 2. A table summarizing the purification of sBMO components is available as supplementary data with the online version of this paper.

Component molecular properties

BMOR. The oxidoreductase component of sBMO exhibited molecular masses of 45 kDa by denaturing SDS-PAGE, as earlier reported (Sluis *et al.*, 2002), and 40.2 kDa by analytical ultracentrifugation. Absorption maxima observed in the UV-visible spectrum were 270, 340, 400 and 458 nm (Fig. 2) and are representative of a flavin and a [2Fe–2S] cluster as has been observed for the reductases of other multiple component monooxygenase systems (Colby & Dalton, 1979; Newman & Wackett, 1995; Patel, 1987). As purified, BMOR showed an $A_{270/458}$ ratio of 3.1 and an $A_{458/340}$ ratio of 1.4. These values were also consistent with other highly purified flavo-iron sulfur proteins (Fox *et al.*, 1989). BMOR displayed a specific activity of 115 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ when catalysing the reduction of 2,6-dichlorophenol indophenol with a 20-fold purification of this activity from the cell-free extract.

BMOB. Previous work on this system reported the requirement of a third component in addition to BMOR

Fig. 1. Denaturing SDS-PAGE of the purified butane monooxygenase components. Lane 1, molecular mass standards (kDa); lane 2, BMOH (5 μg); lane 3, BMOB (2 μg); and lane 4, BMOR (4 μg).

Fig. 2. UV-visible spectra of the purified butane monooxygenase components. BMOR (full line, 1 mg protein ml⁻¹, 25 μ M); BMOB (dot-dash line, 1 mg ml⁻¹, 45 μ M); BMOH (dashed line, 1 mg ml⁻¹, 4 μ M). The inset shows the spectra of the three components from 300 to 550 nm enlarged fourfold.

and BMOH. This third component, presumably BMOB, was found to elute ahead of BMOH during anion-exchange chromatography, but no peptide was found to be enriched in fractions containing this component (Sluis *et al.*, 2002). In this work, BMOB was found to co-elute with BMOH and could be separated by including 1 M KCl during the gel-filtration step of the purification. The identity of BMOB was verified by Edman degradation analysis. Denaturing SDS-PAGE of BMOB revealed an apparent molecular mass of 22 kDa, an unexpected result considering the predicted mass of 15.1 kDa from the deduced amino acid sequence (Sluis *et al.*, 2002). No optical absorbance maxima were observed above 300 nm (Fig. 2), a feature shared by all characterized small components of multicomponent monooxygenase systems (Fox *et al.*, 1989; Green & Dalton, 1985; Newman & Wackett, 1995).

BMOH. The migration of BMOH during gel filtrations indicated a molecular mass between 200 and 260 kDa and, by denaturing SDS-PAGE (Fig. 1), it consisted of three proteins in stoichiometric amounts exhibiting apparent

molecular masses of 54, 43 and 25 kDa as previously observed (Sluis *et al.*, 2002). This result suggests that, like other multicomponent monooxygenase systems, the holoenzyme has an ($\alpha\beta\gamma$)₂ quaternary structure and is consistent with the deduced amino acid sequences of the three subunits of the butane monooxygenase operon. BMOH showed an absorption maximum at 280 nm with no other significant features above 300 nm. This spectrum indicated no contamination by haem or iron-sulfur cluster prosthetic groups of other proteins (e.g. BMOR) in the purified preparations. BMOH was judged pure by denaturing SDS-PAGE and the absence of UV-visible spectral characteristics above 300 nm (Fig. 2). Iron content analysis of BMOH preparations, determined colorimetrically, revealed between 2.8 and 3.6 mol iron (mol BMOH)⁻¹. This iron content translates to 1.4–1.8 diiron clusters per holoenzyme. BMOH specific activities for the epoxidation of ethene in the presence of catalase (see below) were 500–600 nmol min⁻¹ (mg protein)⁻¹.

Catalytic properties

Hydroxylation of substrates under multiple turnover conditions coupled to NADH oxidation required only the BMOR and BMOH components. Maximal activity was reached at a ratio of BMOR to BMOH of 2 : 1 (mol/mol). The addition of BMOB to assays containing BMOR and BMOH resulted in modest increases in rate, with a maximum 20% increase in activity achieved between a ratio of 1.5–2 (mol/mol) BMOB to BMOH. The ratio of BMOB to BMOH that allowed for maximal activity varied between preparations of BMOH. Higher ratios of BMOB resulted in a decrease in the rate of substrate oxidation (data not shown). This characteristic was similar to other small components of multicomponent monooxygenase systems, where rate inhibition occurs when the small component to hydroxylase ratio exceeds 2 (Cadieux *et al.*, 2002; Fox *et al.*, 1989; Green & Dalton, 1985).

The rate of the reaction and yield for ethene oxidation were dependent on the presence of catalase in the reaction. Table 1 shows the results of NADH-coupled ethene oxidation, where NADH (462 μ M) was limiting in the reaction for the purpose of assessing yield. In the absence

Table 1. Ethene oxide yield of NADH-coupled ethene oxidation

Ethene oxide values are in nmol and are the means $\pm$ SD of triplicate analyses.

Catalase present*	Components†	3 min	11 min	Yield (%)‡
No	BMOH + BMOR	112 $\pm$ 9	124 $\pm$ 11	27
	BMOH + BMOR + BMOB	137 $\pm$ 12	151 $\pm$ 7	32
Yes	BMOH + BMOR	175 $\pm$ 13	344 $\pm$ 9	74
	BMOH + BMOR + BMOB	212 $\pm$ 8	384 $\pm$ 19	82

*4000 units per reaction.

†Component concentrations when present: BMOR, 2 μ M; BMOH, 2 μ M; BMOB, 4 μ M.

‡Based on 460 nmol NADH added to start the reaction.

of catalase, the coupling of NADH to ethene oxidation was 40–50 % lower, with the majority of the yield being produced in the first 3 min, and resulted in complete inactivation of ethene oxide production. The addition of catalase resulted in a twofold increase in the rate and 2.5–3-fold increase in the yield for ethene oxidation. The uncoupling of steady-state NADH turnover was shown to produce H_2O_2 during the hydroxylation of methane by sMMO (Zhang & Lipscomb, 2006). For sBMO, this was also the case in the presence of butane. H_2O_2 was produced by the reaction of NADH oxidation to the reduction of O_2 catalysed by BMOR with a yield of $185 \pm 9 \mu\text{M}$ H_2O_2 . When BMOH was present the amount of H_2O_2 at the end of the reaction was $16 \pm 2 \mu\text{M}$, approximately 10 % of that with BMOR alone. Incubation of BMOR with 10 or 100 mM H_2O_2 for 30 min resulted in ethene oxide production rates that were 74 and 46 %, respectively, of those for BMOR incubated without addition of H_2O_2 but did not result in complete inactivation of ethene oxide production as shown in Table 1. Treatment of BMOR with 10 mM H_2O_2 and 5 mM NADH also resulted in decreased ethene oxide production rates but not complete inactivation of this activity. Addition of catalase and fresh BMOR to a reaction without catalase (Table 1) did not result in the restoration of ethene oxide production and showed that BMOH was completely inactivated during turnover in the absence of catalase. The catalase activity of BMOH (see below) made the determination of H_2O_2 yield difficult and thus the values shown should be viewed as a minimum. The presence of BMOB in the reaction had no statistically measurable effect on the yield of H_2O_2 generated ($19 \pm 2 \mu\text{M}$).

Unlike other purified multicomponent monooxygenase systems, BMOB of sBMO does not appear to be required for efficient hydroxylation of substrate (in this study the substrate was ethene) (Cadieux *et al.*, 2002; Fox *et al.*, 1989; Green & Dalton, 1985; Newman & Wackett, 1995). BMOR and BMOH alone account for 80 % of the activity and 90 % product yield during NADH-coupled multiple turnover in the presence of catalase.

H_2O_2 -dependent BMOH activities

The hydroxylase subunits of sMMO and toluene 2-monooxygenase (T2MO) of *Burkholderia cepacia* G4 were

shown to form reactive oxygenating species from H_2O_2 resulting in multiple substrate turnover in the absence of O_2 (Andersson *et al.*, 1991; Froland *et al.*, 1992; Newman & Wackett, 1995). BMOH was also capable of H_2O_2 -dependent substrate turnover, also known as the peroxide shunt reaction. The results demonstrate the dependence of H_2O_2 concentration on the production of ethene oxide and the loss of recoverable BMOH activity in the initial absence of O_2 (Table 2). Reactions containing only BMOR, BMOB, or acetylene-inactivated BMOH did not result in the formation of ethene oxide and no detectable loss of activity was observed when BMOH was incubated without H_2O_2 . The product yield of the peroxidation reaction was three orders of magnitude lower compared to sMMO. A H_2O_2 utilization to product ratio (100 mM H_2O_2 reaction) of approximately 3000 : 1 was observed. As the concentration of H_2O_2 was increased, the recoverable activity of BMOH decreased, but did not result in total inactivation. At the end of the reaction, bubbles were visibly present in the reaction vial, suggesting the evolution of O_2 from H_2O_2 . Experiments confirming that BMOH was catalysing the dismutation of H_2O_2 were carried out utilizing an O_2 electrode. BMOH was observed to follow Michaelis–Menten kinetics for the catalase reaction. The following kinetic parameters were deduced from a Hanes plot of the data: $K_{\text{m}(\text{app})} = 110 \text{ mM}$, $V_{\text{max}} = 5500 \text{ nmol } \text{O}_2 \text{ min}^{-1} (\text{mg hydroxylase})^{-1}$, $k_{\text{cat}} = 46 \text{ s}^{-1}$ and $k_{\text{cat}}/K_{\text{m}} = 417 \text{ s}^{-1} (\text{M})^{-1}$.

Dimerization of BMOB through disulfide bond formation

During gel filtration, BMOB eluted from the column at a molecular mass of approximately 44 kDa. This result indicated that BMOB was eluting from the column as a dimer. The MMOB proteins from *Methylosinus trichosporium* OB3b, *Methylococcus capsulatus* Bath and *Methylocystis* sp. strain M also form dimers under certain conditions (Brandstetter *et al.*, 1999; Chang *et al.*, 1999; Fox *et al.*, 1991; Shinohara *et al.*, 1998). The amino acid sequence of BMOB contains one cysteine residue at position 86. The possibility of disulfide bridge formation between two monomers of BMOB was examined by non-reducing conditions during denaturing SDS-PAGE (Fig. 3). As purified, BMOB migrated at apparent molecular masses of 44 and 22 kDa. Upon reduction with 10 %

Table 2. Ethene oxide production catalysed by BMOH with H_2O_2

The BMOH concentration was 200 μM . Reactions were allowed to proceed for 30 min. Values are means $\pm$ SD of triplicate analyses.

H_2O_2 (mM)	EO produced (nmol)	H_2O_2 remaining (mM)	EO activity remaining (%)
10	ND	0.05 ± 0.04	93 ± 3
50	≤ 10	0.9 ± 0.1	63 ± 7
100	34 ± 8	6.4 ± 3.2	≤ 20

ND, Not detected.

Fig. 3. Denaturing SDS-PAGE of reduced and non-reduced BMOB. Lane 1, molecular mass standards (kDa); lane 2, reduced BMOB (2 µg); lane 3, non-reduced BMOB (2 µg).

2-mercaptoethanol in the sample buffer, BMOB displayed a molecular mass of 22 kDa. Reduction of BMOB with equimolar DTT (prior to addition of non-reducing SDS-PAGE sample buffer) produced the same results as shown (Fig. 3). Samples of reduced BMOB by both methods behaved similarly during SDS-PAGE regardless of heat treatment of the sample before separation. These results showed that a disulfide bridge was formed between two BMOB molecules and the cysteine residue at position 86 was responsible for the dimerization. Reduced BMOB did not show any demonstrable differences in enzymic analyses from the as-purified preparation of BMOB.

Product distribution studies

To determine the specificity and selectivity of sBMO, assays for the oxidation of physiologically relevant linear and branched alkanes were performed and the distribution of products was compared (Table 3). The associated error in the product determinations was $\pm 5\%$ of the values shown. Remarkable similarity existed in the oxidations of the linear alkanes, where 80% or more of the product was observed to be hydroxylated at the terminal carbon. Propane and butane showed the same distributions, with the secondary alcohols, 2-propanol and 2-butanol, respectively, constituting the remaining observed product. The remaining products of pentane oxidation were the secondary alcohols 2-pentanol and 3-pentanol. The small amount of 3-pentanol was not reported in the oxidation of pentane by sMMO (Froland *et al.*, 1992). The addition of the hydroxyl radical scavenger mannitol (50 mM) had no effect on the production of 3-pentanol or the product distribution of pentane oxidation in general. Unlike the sMMO systems, where the addition of MMOB drastically shifts the distribution for most alkanes towards primary hydroxylation (Froland *et al.*, 1992), there was no

Table 3. Substrate range and product distribution of sBMO

Values are means of triplicate analyses; the associated error was within $\pm 5\%$ of the value shown.

Substrate	Product(s)*	Percentage of product†	
		without BMOB	with BMOB‡
Propane	1-Propanol (1°)	82	82
	2-Propanol (2°)	18	18
Butane	1-Butanol (1°)	83	83
	2-Butanol (2°)	17	17
Pentane	1-Pentanol (1°)	80	88
	2-Pentanol (2°)	13	11
	3-Pentanol (2°)	7	1
Hexane	1-Hexanol (1°)	90	86
	2-Hexanol (2°)	10	14
	3-Hexanol (2°)	0	0
Isobutane	2-Methyl-1-propanol (1°)	95	96
	2-Methyl-2-propanol (3°)	5	4
Isopentane	3-Methyl-1-butanol (1°)	63	64
	2-Methyl-1-butanol (1°)	31	31
	3-Methyl-2-butanol (2°)	2	1
	2-Methyl-2-butanol (3°)	4	4

*1°, primary; 2°, secondary; 3°, tertiary.

†Ratio of BMOR to BMOH (2 : 1, mol/mol) for all assays.

‡Ratio of BMOB to BMOH (2 : 1, mol/mol).

significant difference in the shift of products observed when BMOB was present in the reaction.

The strong bias for hydroxylation at the terminal carbon was even more pronounced for the branched alkanes. Isobutane and isopentane both have three terminal carbons and their hydroxylation products result in one and two products, respectively. Terminal products from the oxidation of these branched alkanes by sBMO comprise approximately 95% of the products observed. The remaining products were composed of the other possible alcohols of each respective branched alkane. Again, there was no significant difference in the product distributions when BMOB was present in the reactions for the branched alkanes, revealing that BMOH and BMOR alone were capable of primary hydroxylation.

DISCUSSION

Butane monooxygenase was purified to homogeneity from '*P. butanovora*' and shown to be a soluble, three-component diiron monooxygenase system. As reported previously, the inclusion of Fe^{2+} , glycerol and DTT greatly increased the stability of the hydroxylase during purification procedures (Sluis *et al.*, 2002). The *in vitro* activity for ethene oxidation for the complete monooxygenase system was at least four times the typical *in vivo* activity. The physical characteristics of the purified enzyme components

of sBMO were similar to those of other soluble diiron three-component monooxygenases of this class of diiron monooxygenase. A summary of the reactions possible by sBMO components and multiple component combinations is presented in Table 4.

For NADH-coupled substrate turnover, only BMOH and BMOR were required. In fact, the combination of BMOH and BMOR was capable of efficient ethene turnover at a rate that could account for the *in vivo* rate of ethene oxidation when catalase was present in the reaction. The absence of BMOB in these *in vitro* reactions showed that this component was not required for formation of a reactive oxygen species at the active site for substrate oxidation. Also, BMOB was not required for electron transfer between BMOR and BMOH for the reduction of the active-site irons. BMOB had only modest effects on the NADH-coupled rate of substrate turnover and product yield. The limited influence of BMOB was uncharacteristic for monooxygenase small component proteins, where their addition normally results in 10–150-fold increases in specific activity and ≥ 10 -fold increase in product yield, depending on the substrate being oxidized (Cadieux *et al.*, 2002; Fox *et al.*, 1989; Newman & Wackett, 1995; Wallar & Lipscomb, 1996). BMOH and BMOR were also strongly biased for the terminal hydroxylation of C₃–C₆ linear and branched alkanes, with no statistically significant difference in the product distributions when BMOB was present in the reaction. This result was in contrast to the sMMO small component protein, MMOB. In the absence of MMOB, the oxidation of propane and butane catalysed by the sMMO system was biased for the production of secondary alcohols. When MMOB was included in the reaction, a strong shift in the product distribution toward the primary alcohols was observed (Froland *et al.*, 1992). For the longer linear alkanes, pentane and hexane, sMMO displayed a shift toward primary hydroxylation as the chain length increased and was independent of MMOB. The branched alkane isobutane showed a small shift towards primary hydroxylation with MMOB while with isopentane the shift was more pronounced (Froland *et al.*, 1992).

In the sMMO system, MMOB concentrations $< 5\%$ of MMOH concentrations were responsible for the full effect of MMOB on product distributions during NADH-coupled substrate oxidation (Andersson *et al.*, 1991; Fox

et al., 1989; Froland *et al.*, 1992). Small amounts of contaminating BMOB could explain why there were not large effects on the product distributions for sBMO. Because BMOB was not found in denaturing SDS-PAGE of BMOH preparations, this explanation was not likely for sBMO. Given that there were no dramatic effects attributed to BMOB in the oxidation of substrates, it was imperative to question whether BMOB was interacting with BMOH. The identity of BMOB, given the aberrant migration during denaturing SDS-PAGE, was not in question due to the positive identification by Edman degradation analysis. The copurification of BMOB and BMOH during anion-exchange chromatography and the requirement of high ionic strength needed to separate these two components during gel filtration implied a close association. Dimerization of BMOB due to disulfide-bridge formation between cysteine residues at position 86 was confirmed. Reduction of BMOB prior to inclusion in steady-state ethene turnover experiments displayed the same catalytic properties as the purified preparation. MMOB proteins from the methanotrophs *Methylosinus trichosporium* OB3b, *Methylococcus capsulatus* Bath and *Methylocystis* sp. strain M are also capable of dimer formation under certain conditions (Brandstetter *et al.*, 1999; Chang *et al.*, 1999; Fox *et al.*, 1991; Shinohara *et al.*, 1998). Replacing BMOB in NADH-coupled steady-state ethene turnover reactions with MMOB from *Methylosinus trichosporium* OB3b had similar modest effects on ethene turnover (data not shown). The G113N mutant strain of '*P. butanovora*' contains an amino acid substitution in the α -subunit of BMOH, which is in the area adjacent to or contributing to formation of the active site (Halsey *et al.*, 2006). *In vivo*, '*P. butanovora*' G113N displayed an altered regiospecificity from the wild-type. When butane was supplied as substrate, G113N preferentially hydroxylated the secondary carbon atom, with 2-butanol comprising 92% of the product observed. If BMOB had a role in shifting hydroxylation towards the primary carbon, like MMOB in the sMMO system, such a large shift towards secondary carbon hydroxylation by G113N would not be expected and a more even distribution of products would be observed. The above discussion suggests the unique properties associated with MMOB in the sMMO system have evolved specifically for methane oxidation (Brazeau *et al.*, 2003). Given the rate of ethene oxidation by BMOH and

Table 4. Summary of sBMO component reactions

Component(s)	Substrates	Products
BMOR	NADH + butane + O ₂	H ₂ O ₂
BMOH	H ₂ O ₂	H ₂ O + O ₂
BMOH	H ₂ O ₂ + butane $\pm$ O ₂	1-Butanol + 2-butanol + H ₂ O + O ₂
BMOH + BMOR $\pm$ BMOB	NADH + butane + O ₂	1-Butanol + 2-butanol + H ₂ O ₂ + H ₂ O + O ₂
Catalase + BMOH + BMOR $\pm$ BMOB	NADH + butane + O ₂	1-Butanol + 2-butanol + H ₂ O + O ₂

BMOR in the absence of BMOB, this study suggests that the role of the effector component, BMOB, may be unnecessary for the *in vivo* oxidation of C₂–C₆ aliphatic alkanes.

The inactivation of sBMO during steady-state turnover differed from the sMMO system, where inactivation occurred only in the absence of substrate (Fox *et al.*, 1989). During NADH-coupled turnover, sBMO progressively lost activity, ending in total inactivation, and the presence of substrate provided no protection to this loss. The addition of catalase to the reaction led to a constant rate of product accumulation, which suggested that BMOH and/or BMOR was rapidly inactivated by small amounts of H₂O₂ produced by the uncoupled oxidation of NADH by BMOR in the presence of butane. Pretreatment of BMOR with H₂O₂ (with or without NADH) led to a reduction in the rate of ethene oxide production but not complete inactivation of ethene oxide production. Adding catalase and fresh BMOR to an *in vitro* reaction resulted in complete inactivation of ethene oxide production (Table 1, without catalase). Inactivation of ethene oxide production by H₂O₂ was observed to occur to BMOR and BMOH, though in the case of BMOH inactivation was complete. This observation was not unprecedented for a diiron monooxygenase hydroxylase. The related enzyme system, phenol hydroxylase of *Pseudomonas* sp. strain CF600, was also inactivated in the presence of phenol during NADH-coupled substrate turnover (Cadieux *et al.*, 2002). In contrast, inactivation was not seen in the H₂O₂ shunt reaction, where BMOH in the presence of 10 mM H₂O₂ retained nearly full activity at the end of the assay. Similarly, sMMO and T2MO retained 80 % and 50 %, respectively, of the initial activity after reacting with substrate and 10 mM H₂O₂ for 30 min (Andersson *et al.*, 1991; Newman & Wackett, 1995). Assuming that sBMO follows a similar catalytic cycle as sMMO (Wallar & Lipscomb, 1996), BMOH would be reduced to the diferrous state during NADH-coupled substrate turnover before interacting with O₂. The peroxide shunt mechanism bypasses the diferrous state as H₂O₂ serves as the source of both electrons and O₂. It seemed reasonable to conclude that the rapid inactivation seen during NADH-coupled ethene turnover was dependent on interaction of the diferrous state of BMOH with H₂O₂. This reasoning was bolstered due to the protection afforded by the presence of catalase in the reaction.

This study is the first report of the purification of the enzyme system sBMO from the C₂–C₉ aliphatic alkane oxidizing bacterium '*P. butanovora*'. The reconstituted sBMO complex has been characterized by assessment of NADH coupling to ethene oxidation, H₂O₂ shunt chemistry and product distributions of physiologically relevant linear and branched alkanes. The efficient hydroxylation of ethene and the preferential production of primary alcohols required for downstream catabolism of '*P. butanovora*' was shown to be independent of the small component protein, BMOB. Future experiments are focused on complexation of BMOH and BMOB and methane oxidation utilizing the

site-directed mutants of the α -subunit of the hydroxylase (Halsey *et al.*, 2006).

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REFERENCES

- Andersson, K. K., Froland, W. A., Lee, S. K. & Lipscomb, J. D. (1991). Dioxygen independent oxygenation of hydrocarbons by methane monooxygenase hydroxylase component. *N J Chem* **15**, 411–415.
- Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H. & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int J Syst Evol Microbiol* **50**, 1563–1589.
- Arp, D. J. (1999). Butane metabolism by butane-grown '*Pseudomonas butanovora*'. *Microbiology* **145**, 1173–1180.
- Ashraf, W., Mihdhir, A. & Murrell, J. C. (1994). Bacterial oxidation of propane. *FEMS Microbiol Lett* **122**, 1–6.
- Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein using the principal of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brandstetter, H., Whittington, D. A., Lippard, S. J. & Frederick, C. A. (1999). Mutational and structural analyses of the regulatory protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *Chem Biol* **6**, 441–449.
- Brazeau, B. J., Wallar, B. J. & Lipscomb, J. D. (2003). Effector proteins from P450(cam) and methane monooxygenase: lessons in tuning nature's powerful reagents. *Biochem Biophys Res Commun* **312**, 143–148.
- Cadieux, E., Vrajmasu, V., Achim, C., Powlowski, J. & Munck, E. (2002). Biochemical, Mossbauer, and EPR studies of the diiron cluster of phenol hydroxylase from *Pseudomonas* sp. strain CF 600. *Biochemistry* **41**, 10680–10691.
- Chang, S. L., Wallar, B. J., Lipscomb, J. D. & Mayo, K. H. (1999). Solution structure of component B from methane monooxygenase derived through heteronuclear NMR and molecular modeling. *Biochemistry* **38**, 5799–5812.
- 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). *Biochem J* **177**, 903–908.
- Doughty, D. M., Sayavedra-Soto, L. A., Arp, D. J. & Bottomley, P. J. (2005). Effects of dichloroethene isomers on the induction and activity of butane monooxygenase in the alkane-oxidizing bacterium '*Pseudomonas butanovora*'. *Appl Environ Microbiol* **71**, 6054–6059.
- Doughty, D. M., Sayavedra-Soto, L. A., Arp, D. J. & Bottomley, P. J. (2006). Product repression of alkane monooxygenase expression in *Pseudomonas butanovora*. *J Bacteriol* **188**, 2586–2592.
- Fox, B. G., Froland, W. A., Dege, J. E. & Lipscomb, J. D. (1989). Methane monooxygenase from *Methylosinus trichosporium* OB3b. Purification and properties of a three-component system with high specific activity from a type II methanotroph. *J Biol Chem* **264**, 10023–10033.
- Fox, B. G., Froland, W. A., Jollie, D. R. & Lipscomb, J. D. (1990). Methane monooxygenase from *Methylosinus trichosporium* OB3b. *Methods Enzymol* **188**, 191–202.

- Fox, B. G., Liu, Y., Dege, J. E. & Lipscomb, J. D. (1991).** Complex formation between the protein components of methane monooxygenase from *Methylosinus trichosporium* OB3b. Identification of sites of component interaction. *J Biol Chem* **266**, 540–550.
- Froland, W. A., Andersson, K. K., Lee, S. K., Liu, Y. & Lipscomb, J. D. (1992).** Methane monooxygenase component-B and reductase alter the regioselectivity of the hydroxylase component-catalyzed reactions – a novel role for protein-protein interactions in an oxygenase mechanism. *J Biol Chem* **267**, 17588–17597.
- Funhoff, E. G., Bauer, U., Garcia-Rubio, I., Witholt, B. & van Beilen, J. B. (2006).** CYP153A6, a soluble P450 oxygenase catalyzing terminal-alkane hydroxylation. *J Bacteriol* **188**, 5220–5227.
- Green, J. & Dalton, H. (1985).** Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *J Biol Chem* **260**, 15795–15801.
- Halsey, K. H., Sayavedra-Soto, L. A., Bottomley, P. J. & Arp, D. J. (2005).** Trichloroethylene degradation by butane-oxidizing bacteria causes a spectrum of toxic effects. *Appl Microbiol Biotechnol* **68**, 794–801.
- Halsey, K. H., Sayavedra-Soto, L. A., Bottomley, P. J. & Arp, D. J. (2006).** Site-directed amino acid substitutions in the hydroxylase alpha subunit of butane monooxygenase from *Pseudomonas butanovora*: implications for substrates knocking at the gate. *J Bacteriol* **188**, 4962–4969.
- Hamamura, N., Page, C., Long, T., Semprini, L. & Arp, D. J. (1997).** Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and methane-grown *Methylosinus trichosporium* OB3b. *Appl Environ Microbiol* **63**, 3607–3613.
- Hamamura, N., Stofa, R. T., Semprini, L. & Arp, D. J. (1999).** Diversity in butane monooxygenases among butane-grown bacteria. *Appl Environ Microbiol* **65**, 4586–4593.
- Kopp, D. A. & Lippard, S. J. (2002).** Soluble methane monooxygenase: activation of dioxygen and methane. *Curr Opin Chem Biol* **6**, 568–576.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lidstrom, M. E. (1988).** Isolation and characterization of marine methanotrophs. *Antonie Van Leeuwenhoek* **54**, 189–199.
- Lipscomb, J. D. (1994).** Biochemistry of the soluble methane monooxygenase. *Annu Rev Microbiol* **48**, 371–399.
- McLee, A. G., Kormendy, A. C. & Wayman, M. (1972).** Isolation and characterization of n-butane-utilizing microorganisms. *Can J Microbiol* **18**, 1191–1195.
- Nelson, D. C., Waterbury, J. B. & Jannasch, H. W. (1982).** Nitrogen fixation and nitrate utilization by marine and freshwater *Beggiatoa*. *Arch Microbiol* **133**, 172–177.
- Newman, L. M. & Wackett, L. P. (1995).** Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. *Biochemistry* **34**, 14066–14076.
- Patel, R. N. (1987).** Methane monooxygenase: purification and properties of flavoprotein component. *Arch Biochem Biophys* **252**, 229–236.
- Percival, M. D. (1991).** Human 5-lipoxygenase contains an essential iron. *J Biol Chem* **266**, 10058–10061.
- Perry, J. J. (1980).** Propane utilization by microorganisms. *Adv Appl Microbiol* **26**, 89–115.
- Sayavedra-Soto, L. A., Byrd, C. M. & Arp, D. J. (2001).** Induction of butane consumption in *Pseudomonas butanovora*. *Arch Microbiol* **176**, 114–120.
- Sayavedra-Soto, L. A., Doughty, D. M., Kurth, E. G., Bottomley, P. J. & Arp, D. J. (2005).** Product and product-independent induction of butane oxidation in *Pseudomonas butanovora*. *FEMS Microbiol Lett* **250**, 111–116.
- Shanklin, J., Achim, C., Schmidt, H., Fox, B. G. & Munck, E. (1997).** Mossbauer studies of alkane omega-hydroxylase: evidence for a diiron cluster in an integral-membrane enzyme. *Proc Natl Acad Sci U S A* **94**, 2981–2986.
- Shinohara, Y., Uchiyama, H., Yagi, O. & Kusukabe, I. (1998).** Purification and characterization of component B of a soluble methane monooxygenase from *Methylocystis* sp. M. *J Ferment Bioeng* **85**, 37–42.
- Sluis, M. K., Sayavedra-Soto, L. A. & Arp, D. J. (2002).** Molecular analysis of the soluble butane monooxygenase from '*Pseudomonas butanovora*'. *Microbiology* **148**, 3617–3629.
- Takahashi, J., Ichikawa, Y., Sagae, H., Komura, I., Kanou, H. & Yamada, K. (1980).** Isolation and identification of n-butane-assimilating bacterium. *Agric Biol Chem* **44**, 1835–1840.
- van Beilen, J. B., Smits, T. H., Roos, F. F., Brunner, T., Balada, S. B., Rothlisberger, M. & Witholt, B. (2005).** Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases. *J Bacteriol* **187**, 85–91.
- van Beilen, J. B., Funhoff, E. G., van Loon, A., Just, A., Kysser, L., Bouza, M., Holtackers, R., Rothlisberger, M., Li, Z. & Witholt, B. (2006).** Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. *Appl Environ Microbiol* **72**, 59–65.
- Waller, B. J. & Lipscomb, J. D. (1996).** Dioxygen activation by enzymes containing binuclear non-heme iron clusters. *Chem Rev* **96**, 2625–2657.
- Wiegant, W. W. & deBont, J. A. M. (1980).** A new route for ethylene glycol metabolism in *Mycobacterium* E44. *J Gen Microbiol* **120**, 325–331.
- Zhang, J. & Lipscomb, J. D. (2006).** Role of the C-terminal region of the B component of *Methylosinus trichosporium* OB3b methane monooxygenase in the regulation of oxygen activation. *Biochemistry* **45**, 1459–1469.

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