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ORIGINAL RESEARCH PAPER

The substrate binding cavity of particulate methane monooxygenase from *Methylosinus trichosporium* OB3b expresses high enantioselectivity for *n*-butane and *n*-pentane oxidation to 2-alcohol

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Abstract The particulate methane monooxygenase (pMMO) of *Methylosinus trichosporium* OB3b oxidized *n*-butane and *n*-pentane and mainly produced (*R*)-2-butanol and (*R*)-2-pentanol that comprised 78 and 89% of the product, respectively, indicating that the *pro-R* hydrogen of the 2-position carbon of *n*-butane and *n*-pentane is oriented toward a catalytic site within the substrate binding site of pMMO. The protein cavity adjacent to the catalytic center for pMMO has optimum volume for recognizing *n*-butane and *n*-pentane for enantioselective hydroxylation.

Keywords *n*-Alkane recognition · Particulate methane monooxygenase · Selective hydroxylation

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Introduction

Selective oxidation of *n*-alkanes to alcohol is a challenging reaction. First, *n*-alkanes are difficult to hydroxylate due to their inert C–H bond (Kerr 1966). Additionally, selective production of alcohols from *n*-alkanes is thermodynamically difficult: the activation energies for subsequent oxidations of an alcohol are lower than the energy required for the initial hydroxylation of the starting alkane, resulting in mixtures of alcohol, ketone, aldehyde, and carboxylic acid products by alkane oxidation reactions (Labinger 2004). Selective *n*-alkane oxidation to alcohol is accomplished by some alkane monooxygenases. For instance, methane monooxygenase can selectively convert methane, the most inert alkane, to methanol under ambient conditions.

Alkane monooxygenases have an active site that combines the substrate binding site with a reactive catalytic metal center. The active site of some alkane monooxygenases is capable of binding an alkane molecule in a favorable orientation toward the catalytic center, thus showing regio- and stereoselectivity for alkane hydroxylation (Li and Chang 2004; Funhoff and van Beilen 2007). For alkane orientation, multiple amino acid residues at the substrate binding site play a role in the interaction with the alkane molecule via van der Waals interactions and hydrogen bonding (Mas-Ballesté and Que 2006). Short-chain *n*-alkanes, such as *n*-butane and *n*-pentane, have fewer sites in their molecular structure to

interact with substrate binding site than bulky, long-chain alkanes, and thus are expected to be difficult to orient toward the catalytic center even in the enzyme environment. Enantioselective hydroxylation of *n*-butane and *n*-pentane by monooxygenase has only been reported for particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) ($pMMO^{Bath}$). pMMO convert not only methane but also some *n*-alkanes (C2–C5) and alkenes (C2–C4) as non-growth substrates. When 2-butanol and 2-pentanol are produced from *n*-butane and *n*-pentane by $pMMO^{Bath}$, the *R*-isomers comprise 79 and 89% of the product, respectively. The enantioselectivity of $pMMO^{Bath}$ indicates that *n*-butane and *n*-pentane, which are non-growth substrates for pMMO, are recognized at the substrate binding site of $pMMO^{Bath}$, and are oriented toward the catalytic center of the enzyme in a particular manner (Elliot et al. 1997).

The substrate binding site of pMMO has not been identified. The site is expected to be the cavity or pocket adjacent to the catalytic site in pMMO. Structural information of pMMO from *Methylosinus trichosporium* OB3b ($pMMO^{OB3b}$) and $pMMO^{Bath}$ revealed by X-ray crystallographic analysis is now available (Lieberman and Rosenzweig 2005; Hakemian et al. 2008). A di-nuclear copper center is found at the water-imposed, *N*-terminal domain of PmoB, which is one of three subunits of pMMO. Also, mono-nuclear copper center is found in the inter-membrane subunit, PmoC, which was suggested to be capable of housing mono-nuclear zinc center or di-iron center (Lieberman and Rosenzweig 2005; Martinho et al. 2007). In addition, the hydrophilic space, which was suggested to be the site of a tri-copper cluster, was found within the subunit PmoA (Chan et al. 2007). At least one of these metal centers is likely to be the catalytic site of pMMO, while the number and type of metal centers and location of the catalytic site is still in question.

Based on studies of molecular recognition of synthetic molecular capsules, the cavity volume is proposed to be a good index for identifying cavities that would recognize a guest molecule. Mecozzi and Rebek reported (1998) that a host cavity can recognize a guest molecule with some intermolecular interaction when the packing coefficient, defined as the volume ratio between the guest molecule and the host cavity, is in the range 0.46–0.64 (vol/vol). This value can extend to 0.70 (vol/vol) if the guest–host complex is stabilized by strong intermolecular forces such as hydrogen bonds. If the packing coefficient is above 0.70 (vol/vol), the host cavity cannot house a guest molecule. The concept that cavity volume governs, to a first approximation, the recognition of a host molecule applies in particular to apolar binding processes, not only within a synthetic molecular capsule but also, in some cases, within an enzyme environment (Liu et al. 2005; Zürcher et al. 2008). *n*-Butane and *n*-pentane are apolar molecules, and thus the recognition of these *n*-alkanes may be governed by the volume of the substrate binding site of pMMO.

In the present study, we predicted the volume of substrate binding site in $pMMO^{OB3b}$ from the volume of the substrates oxidized and recognized for expressing the enantioselectivity for *n*-alkane hydroxylation and alkene epoxidation. For the prediction, we applied the concept of molecular recognition proposed by Mecozzi and Rebek. To impose enantioselectivity by pMMO for the oxidation of *n*-butane and *n*-pentane to 2-alcohol, we performed *n*-butane and *n*-pentane oxidation reactions using whole cell pMMO.

Materials and methods

Whole cells of *M. trichosporium* OB3b (ATCC35070) and *M. capsulatus* (Bath) (ATCC33009) were used for the *n*-butane and *n*-pentane oxidation reactions. The cells were grown on a 3 l scale on nitrate mineral salts medium containing 10 μM $CuSO_4$ as described previously (Miyaji et al. 2009). Under these growth conditions pMMO is expressed in the bacterial cells (Park et al. 1992).

Oxidation of *n*-butane and *n*-pentane were carried out using a 50 ml 3-neck flask at 30°C under atmospheric pressure. For *n*-butane oxidation, the flask put under vacuum and then filled with *n*-butane and air provided by balloons. In this flask, 13.5 ml 25 mM MOPS buffer (pH 7.0) containing 20 mM sodium formate was saturated with *n*-butane and air. For *n*-pentane oxidation, *n*-pentane was added to the buffer in a flask filled with air and N_2. To initiate the oxidation, 1.5 ml bacterial cells suspended in 25 mM MOPS buffer (pH 7.0) (50 mg dry cells/ml) was added to the reactor. Samples were taken, immediately placed on ice to quench the reaction, centrifuged briefly to pellet the bacterial cells, and the

supernatant was then applied to GC with a flame ionization detector. Analysis of alcohols was performed on a Thermon-1000 5% Sunpak A column (3.2 mm i.d. × 2 m), while that of aldehydes and ketones was performed on a TSG-1 Shimalite F column (3.2 mm i.d. × 3 m). To determine the enantioselectivity of product formation, the reaction products were extracted into dichloromethane from the reaction mixture, and the organic layer was dried over anhydrous $MgSO_4$. The enantiochemical identity of 2-alcohol in the reaction product was assigned by ^{1}H NMR of (*R*)-*O*-acetylmandelic acid ester derivatives using authentic standards (Yu et al. 2003). The authentic standards were synthesized as reported previously (Yu et al. 2003) and were confirmed by ^{1}H NMR.

Results and discussion

Oxidations of *n*-butane and *n*-pentane were performed at 30°C by whole cell pMMO^{OB3b}. The reaction proceeded at constant rate for 2 h (Fig. 1) as also observed by alkene epoxidation by whole cell pMMO^{OB3b} (Ono and Okura 1990). The product distributions of the reactions over the 2 h are summarized in Table 1. From *n*-butane, 2-butanol was produced, with a product distribution of 90%. Butanol was not detected. 2-Butanone was produced when 2-butanol had accumulated (Fig. 1), suggesting that 2-butanone was generated by 2-butanol oxidation in the whole cells. 2-Pentanol was produced from *n*-pentane and 1-pentanol and 3-pentanol were not detected. *n*-Butane and *n*-pentane oxidation to 2-alcohol should proceed within the pMMO^{OB3b} active site and, within the active site the 2-position of the C–H bond of *n*-pentane is assumed to be positioned close to the catalytic site.

2-Butanol and 2-pentanol are enantiomeric; thus, the enantioselectivity for 2-alcohol from the *n*-butane and *n*-pentane oxidation by whole cells pMMO^{OB3b} was analyzed. As shown in Table 1, the enantioselectivities of the *R*-forms of 2-butanol and 2-pentanol were 78 and 89%, respectively, indicating that pMMO^{OB3b} can oxidize *n*-butane and *n*-pentane enantioselectively to (*R*)-2-alcohol. To produce the *R*-form of 2-alcohol selectively, a *pro-R* hydrogen at the 2-position of *n*-alkanes needs to be oriented toward the catalytic site within the pMMO^{OB3b}

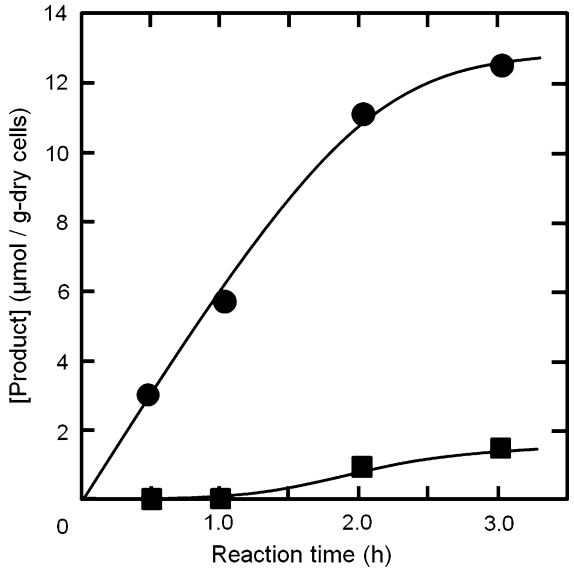

Fig. 1 2-Butanol and 2-butanone production from *n*-butane by whole cells of *M. trichosporium* OB3b expressing pMMO. *Filled circles* 2-butanol, *filled squares* 2-butanone

substrate binding site (Fig. 2). In other words, the pMMO^{OB3b} substrate binding site has the ability to orient *n*-butane and *n*-pentane.

pMMO^{OB3b} can oxidize some alkenes to epoxide enantioselectively as reported by Ono and Okura (1990). pMMO^{OB3b} produces (*S*)-1,2-epoxybutane selectively from 1-butene. In this reaction, the *si*-face of 1-butene is oriented toward the catalytic site of pMMO^{OB3b}. Epoxidation of 1,3-butadiene to (*S*)-1,2-epoxybutene proceeds in the same direction. As shown in Fig. 1, the direction of the *si*-face relative to 1-butene and 1,3-butadiene carbon chains is the same as that of *pro-R* hydrogen relative to an *n*-butane carbon chain, indicating that the 1- to 3-position carbons of 1-butene, *n*-butane, and *n*-pentane tend to be oriented in the same direction within the pMMO^{OB3b} substrate binding site. On the other hand, propene epoxidation proceeds mostly racemically. The substituted group at the 3-position carbon of *n*-alkane or alkene may contribute to the enantioselectivity.

According to the proposal of Mecozzi and Rebek (1998), a cavity of host molecule cannot house a guest molecule when the packing coefficient is above 0.7 (v/v). The substrate binding site of pMMO^{OB3b} can house *n*-pentane (96.4 Å^3) (Table 2) but not *n*-hexane (120.4 Å^3) (Burrows et al. 1984), indicating that the volume of *n*-pentane is the largest volume

Table 1 *n*-Butane and *n*-pentane oxidation by whole cells of *M. trichosporium* OB3b and *M. capsulatus* (Bath) expressing pMMO

Strain	Substrate	Product	Product amount (μmol/g dry cells)[a]	Product distribution (%)	Selectivity for *R*-form (%)
$pMMO^{OB3b}$	*n*-Butane	2-Butanol	11	92	78
		2-Butanone	0.9	8	–
	n-Pentane	2-Pentanol	3.5	100	89
$pMMO^{Bath}$	*n*-Butane	2-Butanol	92	90	78
		2-Butanone	10	10	–
	n-Pentane	2-Pentanol	14	100	89

[a] Product amount of the reactions over 2 h

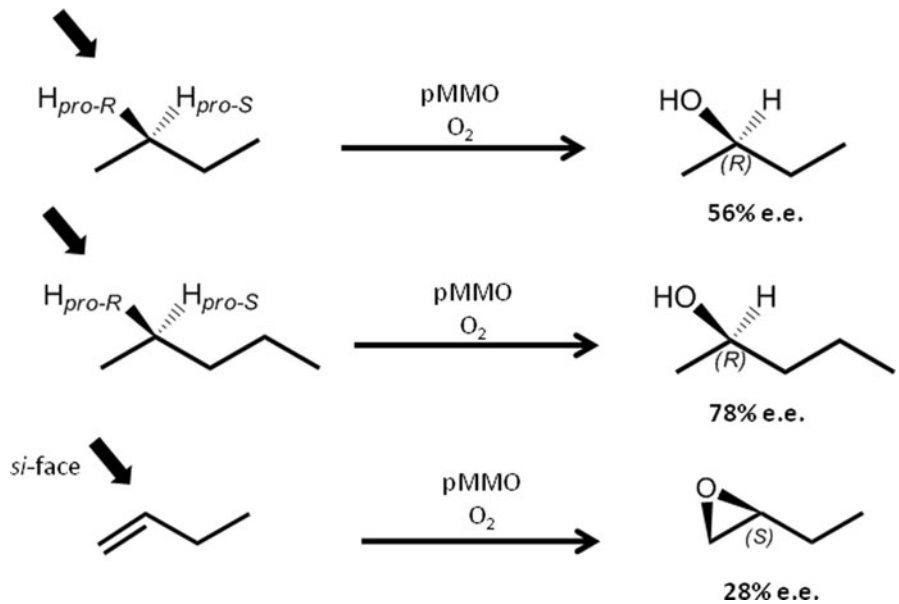

Fig. 2 Orientation of *n*-butane, *n*-pentane, and 1-butene toward the catalytic site within the $pMMO^{OB3b}$ active site. The *arrows* indicate the direction of the catalytic site within the pMMO active site

that the substrate binding site of $pMMO^{OB3b}$ can house. By assuming that the packing coefficient of *n*-pentane in the substrate binding site of pMMO is ~0.7 (v/v), the volume of pMMO substrate binding site is calculated to be ~138 $Å^3$.

The volume of the $pMMO^{OB3b}$ substrate binding site predicted above reasonably explains the expression of enantioselectivity for *n*-alkane hydroxylation and alkene epoxidation. In Table 2, the enantioselectivities of $pMMO^{OB3b}$ for *n*-alkane hydroxylation to (*R*)-2-alcohol, and alkene epoxidation to (*S*)-1,2-epoxyalkane (Ono and Okura 1990), are summarized along with the packing coefficients of substrates within the putative $pMMO^{OB3b}$ substrate binding site. When the enantioselectivity of $pMMO^{OB3b}$ is expressed, the packing coefficient is in the range of 0.46–0.70 (vol/vol). Therefore, the cavity reasonably explains the recognition of those substrates to express enantioselectivity. The importance of cavity volume for expressing product selectivity has also been demonstrated in some reactions within pores of zeolites (Corma et al. 1997; Koyama et al. 2010). Compared with zeolite pore, a protein cavity of pMMO is expected to have more flexible structure. However, the cavity in pMMO where non-growth substrate is accommodated may be rigid.

n-Butane and *n*-pentane oxidation reactions were also performed using whole cell $pMMO^{Bath}$. The product distributions of the reactions were almost the same as that by whole cell $pMMO^{OB3b}$ (Table 1), and

Table 2 Stereoselectivities of $pMMO^{OB3b}$ for alkane hydroxylation and alkene epoxidation

Substrate	Molecular volume ($Å^3$)[a]	Packing coefficient (v/v)[b]	Dominant enantiomer	Enantioselectivity (% ee)	Reference
Propene	56.6	0.41	(*R*)-1,2-Epoxypropane	6	Ono and Okura (1991)
1,3-Butadiene	67.8	0.49	(*S*)-1,2-Epoxybutene	28	Ono and Okura (1991)
1-Butene	73.6	0.53	(*S*)-1,2-Epoxybutane	28	Ono and Okura (1991)
n-Butane	79.4	0.58	(*R*)-2-Butanol	56	This study
n-Pentane	96.4	0.70	(*R*)-2-Pentanol	78	This study

[a] Van der Waals volume calculated using values reported by Bondi (1964)

[b] Molecular volume of substrate divided by volume of the substrate binding site in $pMMO^{OB3b}$ predicted here (138 $Å^3$)

were also the same as reported previously (Elliot et al. 1997). This result suggests that the volume of the substrate binding site is almost the same between $pMMO^{OB3b}$ and $pMMO^{Bath}$.

The cavity volume predicted here is too large to explain the specificity of pMMO for methane (28.4 $Å^3$), the physiological substrate. According to the study by Sugimori et al. (1995), the Michaelis–Menten constant of $pMMO^{OB3b}$ for propane is drastically larger than that for methane and ethane, suggesting that the binding mode at the substrate binding site with methane and at that with the *n*-alkanes larger than propane differ. Presumably, the substrate binding cavity changes its structure for stabilizing the transition state of methane, as explained by the induced-fit mechanism (Koshland 1958). On the other hand, we also presumed that the cavity shows little structural changes like synthetic molecular capsule when non-growth substrate is accommodated, because the expression of enantioselectivity can be explained by the molecular recognition mechanism of rigid synthetic molecular capsule. From these two distinct behaviors of the substrate binding cavity, we hypothesize two substrate binding sites in pMMO, which was also proposed as the substrate binding site model of ammonia monooxygenase of *Nitrosomonas europaea* (Keener and Arp 1993), a homologue of pMMO: one site for small substrate such as methane and ethane, and the other for larger non-growth substrates than ethane such as *n*-butane and *n*-pentane. If the two sites are located in one cavity of pMMO, the site for methane may play a role in inducing the structural change of the cavity. Non-growth substrate cannot bind at the site for methane. Therefore, the cavity does not change its structure when the substrate binding cavity houses a non-growth substrate. Alternatively, there are two distinct cavities in a pMMO molecule, or there are two distinct conformations of pMMO with one substrate binding cavity as explained by the population shift mechanism (Okazaki and Takada 2008). According to these models, the volume we predicted is that of the cavity for non-growth substrate, but not for methane.

In summary, $pMMO^{OB3b}$ has the ability to produce (*R*)-2-alcohol from *n*-butane and *n*-pentane enantioselectively. Assuming that the proposed packing coefficient does predict molecular recognition of a synthetic molecular capsule, the volume of the substrate binding cavity is estimated to be $\sim$138 $Å^3$ from the molecular volume of *n*-pentane, the largest substrate for $pMMO^{OB3b}$. The volume is reasonable for recognizing *n*-butane, *n*-pentane, and 1-butene to produce 2-alcohol or epoxide enantioselectively.

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