Propionate inactivation of butane monooxygenase activity in '*Pseudomonas butanovora*': biochemical and physiological implications

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Butane monooxygenase (BMO) catalyses the oxidation of alkanes to alcohols in the alkane-utilizing bacterium '*Pseudomonas butanovora*'. Incubation of alkane-grown '*P. butanovora*' with butyrate or propionate led to irreversible time- and O₂-dependent loss of BMO activity. In contrast, BMO activity was unaffected by incubation with lactate or acetate. Chloramphenicol inhibited the synthesis of new BMO, but did not change the kinetics of propionate-dependent BMO inactivation, suggesting that the propionate effect was not simply due to it acting as a repressor of BMO transcription. BMO was protected from propionate-dependent inactivation by the presence of its natural substrate, butane. Although both the time and O₂ dependency of propionate inactivation of BMO imply that propionate might be a suicide substrate, no evidence was obtained for BMO-dependent propionate consumption, or ¹⁴C labelling of BMO polypeptides by [2-¹⁴C]propionate during inactivation. Propionate-dependent BMO inactivation was also explored in mutant strains of '*P. butanovora*' containing single amino acid substitutions in the α -subunit of the BMO hydroxylase. Propionate-dependent BMO inactivation in two mutant strains with amino acid substitutions close to the catalytic site differed from wild-type (one was more sensitive and the other less), providing further evidence that propionate-dependent inactivation involves interaction with the BMO catalytic site. A putative model is presented that might explain propionate-dependent inactivation of BMO when framed within the context of the catalytic cycle of the closely related enzyme, soluble methane monooxygenase.

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INTRODUCTION

Bacterial monooxygenase enzymes have been extensively studied because of their potential to degrade environmental pollutants, and to serve as biocatalysts for the creation of industrially important chemicals (Astier *et al.*, 2003; Lipscomb, 1994; Smith *et al.*, 2003; Smith & Dalton, 2004). In this connection we have studied the transcriptional and physiological regulation of butane monooxygenase (BMO) in '*Pseudomonas butanovora*'. BMO oxidizes n-alkanes C₂ to C₉ to alcohols, which are subsequently metabolized to fatty acids (Arp, 1999; Doughty *et al.*, 2006). We have shown that the activity of the *bmo* promoter is induced by alcohol, aldehyde and epoxide products of alkane and alkene oxidation, and repressed by

micromolar concentrations of propionate (Doughty *et al.*, 2005, 2006; Sayavedra-Soto *et al.*, 2001, 2005). Furthermore, although the organic acids acetate, propionate, butyrate and lactate can act as exogenous sources of reductant to stimulate BMO activity, propionate and butyrate do not sustain linear rates of BMO activity for as long as equivalent amounts of lactate or acetate (Doughty *et al.*, 2005). In this study our observations were extended to show that propionate and butyrate inactivate pre-existing BMO.

BMO shares a high level of amino acid sequence identity with the well-characterized soluble methane monooxygenase (sMMO) found in methanotrophic bacteria (Sluis *et al.*, 2002). Inhibitors and inactivators of sMMO include (i) suicide substrates such as terminal alkynes, which covalently bind to active-site residues following a catalytic transformation in the enzyme's active site (Prior & Dalton, 1985); (ii) heavy metals (e.g. Cu²⁺) that rapidly and

Abbreviations: BMO, butane monooxygenase; MMOH, methane monooxygenase hydroxylase; sMMO, soluble methane monooxygenase.

irreversibly inhibit the reductase subunit of the enzyme, preventing electron transfer to the hydroxylase subunit (Green *et al.*, 1985; Jahng & Wood, 1996); and (iii) H_2O_2 , which inactivates the enzyme through an unknown mechanism (Astier *et al.*, 2003). Because propionate lacked structural similarity to any of the known inhibitors and inactivators of sMMO mentioned above, we thought it worthwhile to characterize the effect of propionate on BMO. As the project evolved, it became clear that intermediate chain-length fatty acids might be potentially useful for further studies focused on dissecting the catalytic mechanism of BMO.

METHODS

Bacterial strains, growth conditions and enzyme assays. Wild-type and mutant strains of '*P. butanovora*' (ATCC 43655) were grown as described previously (Arp, 1999; Hamamura *et al.*, 1999; Vangnai & Arp, 2001). Cells were harvested by sedimentation in a centrifuge (6500 *g* for 10 min), and washed three times in 50 mM sodium/potassium phosphate buffer, pH 7.1. Lactate-supported, ethene-dependent ethene oxide production is a convenient measure of BMO activity. Ethene oxide assays were performed as described previously (Doughty *et al.*, 2005) except that 0.5 ml samples of the headspace were taken at 2.5 min intervals and injected into separate 7 ml sealed vials for later analysis. This variation of the assay allowed for the prompt sampling of a large number of vials and separated experimental execution from the analysis of gas samples. Protein concentrations were determined using the micro-biuret assay described previously (Gornall *et al.*, 1949).

In vivo inactivation of BMO in '*P. butanovora*'. Butane-grown cultures of '*P. butanovora*' were harvested and washed as described above. Vials (160 ml) containing 80 ml of cells (OD_{600} 0.5; 0.2 mg protein ml^{-1}), resuspended in 50 mM sodium/potassium phosphate buffer, served as the reaction chambers for the assays. Vials received 10 mM lactate as an exogenous reductant to support BMO activity and 10 mM of either propionate, butyrate or acetate. Assay mixtures were shaken on an orbital shaker at 200 r.p.m. and 30 °C. Accurate assessment of time-dependent inactivation of BMO required the prompt interruption of the assay. To accomplish this, aliquots of cells were injected into 'stop vials' (160 ml) containing 10 ml 50 mM sodium/potassium phosphate buffer in equilibrium with headspace containing 0.4 mmol butane gas (approx. 200 μM aqueous concentration), which

prevented further inactivation of the BMO enzyme during the washing procedure. Cells were washed three times by centrifugation (6500 *g* for 10 min), resuspended in 50 mM sodium/potassium phosphate buffer, and tested for residual BMO activity using the lactate-dependent ethene oxidation assay described above.

Because substrate oxidation by BMO is dependent upon O_2 as a co-substrate, we also examined the effect of propionate on BMO under anoxic conditions. Vials (160 ml) containing 30 ml of 50 mM sodium/potassium phosphate buffer were sealed with butyl rubber stoppers and made anoxic by flushing the headspace with argon for 2 min. Stock solutions of lactate and propionate, as well as cell suspensions, were prepared using the same method. Lactate (10 mM), propionate (10 mM) and cell suspensions were injected into the reaction vial with an airtight syringe. Aliquots were removed from each treatment and injected into 'stop' vials, washed three times, and tested for BMO activity as described earlier.

Investigation of propionate-dependent inactivation in mutant strains of '*P. butanovora*' with modified BMO hydroxylase.

Amino acid sequence alignments were used to compare the hydroxylase subunit of BMO with the hydroxylase subunit (MMOH) of the well-characterized sMMO enzymes of *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (Halsey *et al.* 2006). Amino acid residues were identified that were likely associated with (a) the active site, (b) entry of substrate into the active site, or (c) the interaction between the hydroxylase subunit and a regulatory subunit, BmoB. These amino acid residues were targeted for site-directed mutagenesis and are described in Table 1. The mutants were screened for sensitivity to propionate as described above.

Labelling studies with $^{14}\text{C}_2\text{H}_2$ and $[2-^{14}\text{C}]\text{propionate}$. $^{14}\text{C}_2\text{H}_2$ was synthesized from $\text{Ba}^{14}\text{CO}_3$ [57 mCi mmol^{-1} (2.1 GBq mmol^{-1})] as described previously (Hyman & Arp, 1992), and $[2-^{14}\text{C}]\text{propionate}$ [50 mCi mmol^{-1} (1.8 GBq mmol^{-1})] was purchased from American Radiolabelled Chemicals. Butane-grown cells were harvested and washed with 50 mM sodium/potassium phosphate buffer containing 50 μg chloramphenicol ml^{-1} to stop the synthesis of protein. Vials (2 ml) containing 200 μl of resuspended cells (approx. 140 μg protein) received 10 mM lactate to serve as an electron donor for BMO activity and 0.2 μmol (1 mM) of either $[2-^{14}\text{C}]\text{propionate}$ or $^{14}\text{C}_2\text{H}_2$. All treatments were incubated for 30 min in a covered water bath shaker (30 °C and 150 r.p.m.). Following incubation, cells were washed three times in 50 mM sodium/potassium phosphate buffer and lysed by the addition of SDS and DTT (Sambrook *et al.*, 1989). Protein (20 μg) from each treatment was loaded onto a 12.5 %

Table 1. Summary of mutant strains of '*P. butanovora*' in which single amino acid substitutions were made to the α -subunit of the BMO hydroxylase (BMOH)

For further details of mutants see Halsey *et al.* (2006).

Strain	Targeted amino acid in BMOH- α *	Corresponding amino acid in MMOH- α	Targeted region of amino acid substitution in BMOH- α
T148C	Thr 148	Cys 151	Near active site
G113N	Gly 113	Asn 116	Near active site
L279F	Leu 279	Phe 282	Hydrophobic cavity leading to active site
F321Y	Phe 321	Tyr 324	Surface of BMOH-believed to be involved in interaction with BMOB
Q320K	Glu 320	Lys 323	Surface of BMOH-believed to be involved in interaction with BMOB

*The presumed position of amino acids is based upon homology to the MMOH- α subunit for which the crystal structure has been determined (Sazinsky & Lippard, 2005).

SDS-PAGE gel and resolved at 15 mA. SDS-PAGE gels were stained with Coomassie blue to visualize the protein, destained and dried in a vacuum desiccator. The incorporation of ^{14}C label was visualized by phosphorimaging and the relative signal densities determined with the ImageQuant software provided by the manufacturer (Molecular Dynamics). A standard curve was prepared by diluting different amounts of $^{14}\text{C}_2\text{H}_2$ -labelled cell extracts into unlabelled cells extracts and comparing the intensities of the images obtained from the standards with the ^{14}C -labelled experimental treatments.

Because complete inactivation of BMO did not occur following incubation with propionate, an experiment was carried out to discriminate between inactivation of a fraction of BMO versus a reduced turnover rate by all BMO molecules. The effect of propionate inactivation on $^{14}\text{C}_2\text{H}_2$ label incorporation into BMO was examined. Butane-grown cells, washed and treated as described above, were exposed to either 10 mM lactate or 10 mM lactate plus 10 mM propionate for 2 h. Cells were washed and aliquots of each treatment were tested for BMO activity or incubated with 10 mM lactate plus $^{14}\text{C}_2\text{H}_2$ for 2 h. Following incubation with $^{14}\text{C}_2\text{H}_2$, whole-cell protein was examined by SDS-PAGE and the incorporation of ^{14}C into the α -subunit polypeptide of BMO hydroxylase determined as described above.

Propionate inactivation of monooxygenases from other bacteria. *Burkholderia cepacia* G4 and *Pseudomonas mendocina* KR-1 were grown in the same medium as described for '*P. butanovora*' with toluene (1 mM) as the carbon source. Cultures were shaken at 200 r.p.m. on an orbital shaker at 30 °C. The methanotrophic bacteria *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), were grown under copper-limiting conditions in a KNO_3 minimal salts medium to promote the production of sMMO rather than pMMO (Lee *et al.*, 2006; Whittenbury *et al.*, 1970). *M. capsulatus* (Bath) was incubated at 37 °C and *M. trichosporium* OB3b was incubated at 30 °C on an orbital shaker at 200 r.p.m. Monooxygenase activity was monitored with the ethene oxide assay described above.

RESULTS

In vivo organic-acid-dependent inactivation of BMO

Doughty *et al.* (2005) showed previously that acetate, propionate and butyrate are effective exogenous sources of reductant for supporting BMO activity in propane-grown cells of '*P. butanovora*'. In this work it can be clearly seen that propionate (10 mM) or butyrate (10 mM) quickly and effectively reduced lactate-dependent BMO activity (Fig. 1). In contrast, the rate of lactate-dependent BMO activity was not affected when acetate (10 mM) or an equivalent volume of phosphate buffer was added to the assay. The kinetics of propionate inactivation of BMO were unchanged even at concentrations as low as 0.1 mM, suggesting that the effect of propionate on BMO reaches saturation at concentrations below 0.1 mM. The same organic acids were tested to determine if the BMO inactivation was reversible or irreversible (Fig. 2). Propionate and butyrate irreversibly inactivated BMO in a time-dependent manner, and BMO activity was not completely restored when propionate or butyrate were removed (Fig. 2). In contrast, BMO activity did not decrease in cells treated with either lactate or a combination of lactate

Fig. 1. Time-course of the effect of butyrate, propionate and acetate on the kinetics of lactate-dependent ethene oxidation. At time zero, vials containing propane-grown cells received 95 μM ethene, and 10 mM lactate was provided as an exogenous source of reductant. At the time indicated by the arrow, vials received either 10 μmol (10 mM in the assay vial) acetate (■), propionate (○) or butyrate (□), or an equivalent volume (10 μl) of buffer (●). Data points are the means of three replicates and error bars represent the standard deviation of the means.

and acetate. From data in Fig. 1 it was calculated that propionate had reduced BMO activity by 73–80% of its initial rate between 5 and 7.5 min after propionate addition. In contrast, Fig. 2 shows that only 40% of BMO was irreversibly inactivated following a similar length of incubation with propionate. These data suggest that propionate acts as both a reversible inhibitor and irreversible

Fig. 2. Time-course of the irreversible effects of 10 mM lactate (●), or a combination of 10 mM lactate and 10 mM acetate (■), propionate (○) or butyrate (□) on BMO activity. Cells were incubated for the length of time indicated on the x-axis, harvested, washed, and tested for residual BMO activity. Data points are the means of three replicates and error bars represent the standard deviation of the means.

inactivator of BMO. The remainder of this study focused on the irreversible effect of propionate on BMO activity.

The presence of butane and the absence of O₂ protect BMO activity from the effect of propionate

A series of experiments was carried out to characterize propionate-dependent BMO inactivation. When butane (0.2 mM) was present in the assay, propionate (10 mM) did not lead to a significant loss of BMO activity (Fig. 3a). Because BMO hydroxylation of butane is dependent upon O₂ as a co-substrate we thought it worthwhile to determine if O₂ was required for propionate-dependent BMO inactivation. Under anoxic conditions, propionate (10 mM) failed to inactivate BMO (Fig. 3b). In contrast, when 22 nmol O₂ was supplied to anoxic cells, the ability of propionate to inactivate BMO was restored. Control

treatments which received either no O₂, or O₂ and no propionate, did not show a significant decrease in BMO activity. Propionate-inactivated BMO did not regain activity following subsequent incubation under anoxic conditions, indicating that BMO activity was protected by anoxia, rather than restored during anoxic treatment. The O₂-dependence of inactivation suggests that the catalytic cycle of BMO is involved in propionate-dependent inactivation.

Determining the effect of protein synthesis on BMO activity

Because the relative rates of BMO synthesis and turnover are unknown, we explored the possibility that propionate-dependent BMO inactivation was due to repression of BMO synthesis. Chloramphenicol (50 µg ml⁻¹) prevented the induction of BMO in repressed lactate-grown cells that were exposed to butane (data not shown). The kinetics of propionate-dependent inactivation of BMO were the same in chloramphenicol-treated and control cells, indicating that the decrease in BMO activity following exposure to propionate was mediated on pre-existing BMO.

[2-¹⁴C]Propionate and ¹⁴C₂H₂ labelling experiments

The possibility that propionate might covalently bind to BMO during inactivation was explored with [2-¹⁴C]propionate. Although SDS-PAGE analysis of cellular proteins following treatment with [2-¹⁴C]propionate showed weak labelling of a few polypeptides, the molecular masses of ¹⁴C-labelled bands did not correspond to the molecular masses of BMO polypeptides. The possibility was considered that propionate might label BMO in an SDS-labile manner. When cell extracts were electrophoresed on native polyacrylamide gels, they also failed to show any association of [2-¹⁴C]propionate with polypeptides of molecular masses equivalent to BMO hydroxylase or reductase (data not shown). These data suggest that [2-¹⁴C]propionate did not covalently bind to the BMO enzyme, and that propionate inactivation did not follow the model of a classical monooxygenase suicide substrate such as acetylene. Furthermore, we explored the possibility that propionate was oxidized by BMO. Propionate consumption by butane-grown '*P. butanovora*' was unaffected by acetylene within the limits of detection [(5 nmol propionate min⁻¹ (mg protein)⁻¹)]. These data further suggest that propionate is not a substrate of BMO.

Because propionate did not completely inactivate BMO, we attempted to distinguish between reduced BMO activity being due to inactivation of a fraction of BMO molecules versus a reduced rate of turnover of all BMO molecules. Butane-grown cells incubated with propionate for 2 h retained ~10% of BMO activity, and all remaining BMO activity was found to be acetylene sensitive (Fig. 4a, b). Furthermore, the incorporation of ¹⁴C from ¹⁴C₂H₂ into

Fig. 3. Independently conducted experiments in which the irreversible inactivation of BMO by propionate was prevented by the inclusion of the enzyme's natural substrate, butane (a), or the removal of O₂ (b), a co-substrate necessary for BMO catalytic turnover. Incubations were 30 min in length and lactate (10 mM) was provided as an exogenous reductant. Data points are the means of three replicates and error bars represent the standard deviation of the means.

Fig. 4. $^{14}\text{C}_2\text{H}_2$ labelling of propionate-treated cells. (a) Ethene oxide production by butane-grown cells (●), and by butane-grown cells that were treated previously for 30 min with either lactate (■), or lactate and propionate (○). (b) Ethene oxide production by the same cells following an additional 2 h incubation with acetylene. (c) $^{14}\text{C}_2\text{H}_2$ -dependent labelling of the α -subunit of the BMO enzyme in cells treated as described in (a), washed, and treated for 2 h with $^{14}\text{C}_2\text{H}_2$.

the 58 kDa polypeptide of the α -subunit of BMO hydroxylase was reduced by ~90 % relative to the buffer control, and was consistent with the propionate-dependent reduction of BMO activity (Fig. 4c). These data suggest that propionate caused BMO inactivation rather than an overall reduction in the rate of BMO turnover.

Inactivation of BMO in '*P. butanovora*' mutants strains containing specific amino acid substitutions in the α -subunit of the BMO hydroxylase

Mutant strains of '*P. butanovora*' that contain single amino acid substitutions in the α -subunit of BMO hydroxylase (BMOH- α) were examined for propionate-dependent inactivation of BMO (Table 2). The effects of propionate on BMO differed among the mutant strains. For example, in the case of the G113N mutant, in which a glycine was replaced by an asparagine residue in a region adjacent to, or contributing to, the active site, BMO activity was not inactivated by propionate. In contrast, mutant strain T148C, in which a threonine in the active-site region was replaced by a cysteine, showed a significant increase in propionate-dependent inactivation of BMO relative to wild-type. Mutants F321Y and L279F, which were modified at amino acid positions believed to be involved in the interaction of BMOH- α with BMOB, were inactivated by propionate to a similar extent as wild-type. Interestingly, reversible inhibition of BMO was observed in all mutant strains, including the G113N mutant. Inhibition of BMO activity by propionate in the G113N mutant was time-dependent, and a 10 min incubation with propionate was sufficient to inhibit ~50 % of BMO activity. Propionate-treated G113N mutant regained BMO activity equivalent to the untreated cells following the washing procedure.

Survey of other monooxygenases for propionate-dependent inactivation

We explored the possibility that other well-studied monooxygenase enzymes that are closely related to BMO might be inactivated by fatty acids. *In vivo* studies showed that the diiron methane monooxygenases (sMMO) of *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, and the toluene *o*-monooxygenase of *Burkholderia cepacia* G4, were not inactivated by propionate. In contrast, the toluene 4-monooxygenase (T_4MO) of *Pseudomonas mendocina* KR1 was inactivated following a

Table 2. Effect of propionate on BMO activity in '*P. butanovora*' mutant strains in the α -subunit of BMO hydroxylase

' <i>P. butanovora</i> ' strain	Sp. act*	BMO activity remaining (%)†	
		Control‡	Propionate treated§
Wild-type	155	96 (15)	41 (7)
G113N	44	92 (20)	103 (16)
T148C	98	102 (11)	15 (3)
L279F	101	90 (9)	56 (8)
F321Y	163	97 (9)	61 (5)
Q320K	56	104 (8)	28 (3)

*Rate of lactate-dependent ethene oxide formation [nmol min^{-1} (mg protein^{-1})] before propionate treatment.

†Values represent the mean of three replicates; the standard deviations of the means are shown in parentheses.

‡Cells were incubated in 50 mM phosphate buffer with 10 mM lactate for 30 min.

§Cells were incubated in 50 mM phosphate buffer with 10 mM lactate and 10 mM propionate for 30 min.

10 min incubation with 10 mM propionate or butyrate by about 60 and 94 % respectively. In contrast, acetate or lactate did not inactivate T₄MO activity, indicating that T₄MO was sensitive to the same range of organic acids as BMO. Furthermore, T₄MO activity was protected from propionate-dependent inactivation by toluene, the physiological substrate of T₄MO (data not shown).

DISCUSSION

The current study provided an unexpected and novel finding, that intermediate chain-length fatty acids inactivate BMO. Because some, but not all, of the BMO activity could be recovered when cells were washed to remove propionate, we were able to provide convincing evidence that both reversible inhibition and inactivation of BMO occurred. Furthermore, propionate-dependent inactivation of BMO was not observed in one mutant strain of '*P. butanovora*' in which glycine 113 (thought to be close to the catalytic site on the α -subunit of BMO hydroxylase) was changed to an asparagine residue. Because the reversible inhibition by propionate remained unaffected in this mutant, these data indicated that reversible inhibition and irreversible inactivation of BMO occur through separate mechanisms.

Propionate inhibition of BMO ties in well with the recent discovery that propionate represses the transcription of BMO genes (Doughty *et al.*, 2006). We previously discussed a model of BMO transcriptional regulation to explain how fatty acid products of alkane oxidation might repress BMO. This form of feedback inhibition resembles the regulation of lipid synthesis (Fab) and fatty acid oxidation (Fad) characterized in *Escherichia coli* (Cronan & Subrahmanyam, 1998). In this connection it is interesting to note that three Fab enzymes are not only repressed by long-chain acyl-CoAs at the level of transcription, but also controlled post-translationally through feedback inhibition by acylated acyl-carrier proteins (acyl-ACP) (Heath & Rock, 1996; Marrakchi *et al.*, 2002). The net result is close coupling of the reductant-consuming lipid biosynthesis pathway with reductant-generating fatty acid β -oxidation. Similarly, the inhibition of alkane oxidation by the downstream products of BMO activity could prevent the allocation of reductant to BMO under circumstances where the production of fatty acids via alkane oxidation exceeds the carbon and energy requirements of the cell. A physiological role for inactivation of BMO can also be envisaged, particularly if the monooxygenase turns over slowly, generates toxic H₂O₂ in the absence of substrate, and also continues to consume reductant.

Because we could not detect propionate consumption during BMO inactivation, and [2-¹⁴C]propionate label did not associate with the BMO polypeptides after electrophoresis under denaturing conditions, propionate inactivation did not follow the model of a monooxygenase suicide substrate such as acetylene. In the context of

existing literature on sMMO, two possible mechanisms for propionate-dependent inactivation of BMO can be proposed. First, *in vitro* studies showed that when sMMO hydroxylase is electrically reduced at the surface of an electrode, O₂ is reduced to H₂O₂ by the hydroxylase and subsequently inactivates it (Astier *et al.*, 2003). We propose a model of BMO inactivation in which propionate stimulates the production of H₂O₂ by BMO and causes oxidative enzyme damage. Propionate-dependent formation of H₂O₂ by BMO would reconcile our observations that BMO inactivation is O₂- and turnover-dependent, while propionate oxidation per se does not occur. A model based upon the catalytic cycle of sMMO is shown in Fig. 5 (Brazeau *et al.*, 2001; Lee *et al.*, 1993a, b; Liu *et al.*, 1995; Zhang & Lipscomb, 2006). The diferric active site, shown at the bottom of the model, is reduced and used to break one bond of O₂, forming intermediate P. The scission of the second O₂ bond results in the formation of intermediate Q. Alternatively, the reaction of the reduced form of sMMO with O₂ can result in H₂O₂ formation and a return of sMMO to a diferric resting state (Zhang & Lipscomb, 2006). Although the presence of the regulatory subunit (MMOB) eliminates the production of H₂O₂ by sMMO hydroxylase, H₂O₂ production resumed, as did enzyme inactivation, following the introduction of substrate to the reaction mixture (Astier *et al.*, 2003). In this context, the aliphatic tail of propionate might resemble the alkane substrate of BMO sufficiently well to reduce the coupling of O₂ activation to substrate oxidation and result in H₂O₂ formation. In this connection there is some indication that BmoB is not involved in the coupling of O₂ activation to substrate turnover by BMO (Dubbels *et al.*, 2007). In methane monooxygenase hydroxylase (MMOH), multiple substrate-binding sites have been proposed for substrates larger than methane (Sazinsky & Lippard, 2005), raising the possibility that butane and propionate compete

Fig. 5. A model of propionate-dependent inactivation of BMO superimposed upon the catalytic cycle of sMMO (Brazeau *et al.*, 2001). See text for details.

for a site outside the substrate-binding pocket. Conceptually, this could reconcile our observations that (i) butane protects BMO from propionate and (ii) propionate is not oxidized by BMO (as propionate does not enter the active site). Our results, although preliminary, raise the possibility that alternative substrate-binding pockets have replaced the role of the coupling subunit in substrate recognition. Second, propionate may form an ionic bond with the active-site irons, and inactivate the enzyme by blocking the active site. In this context, it is interesting to note that crystallographic data obtained from the diferric form of sMMO showed an unidentified electron density within the hydrophobic substrate-binding pocket. Researchers tentatively identified this structure as acetate, a component of the crystallization buffer, with the carboxyl group of acetate forming a bridging ligand between the diirons (Rosenzweig *et al.*, 1997). Additionally, recent research on a structurally related diiron desaturase used acetate as a molecular mimic for O₂ binding. The authors suggested that acetate produced a carboxyl shift in a glutamate residue, resulting in a change in coordination of an active-site iron (Moche *et al.*, 2003). Interestingly, a similar change was observed following the binding of substrate (acyl-ACP) and it is hypothesized that the observed change in iron coordination increases the reactivity of the active site with O₂ (Moche *et al.*, 2003). Our research, although preliminary and *in vivo*, raises the possibility that propionate could be useful as a probe to uncover some of the mechanistic details of BMO. Because of the novelty of propionate-dependent inactivation, we extended our study to mutant strains of '*P. butanovora*' in which single amino acid substitutions had been made to the α -subunit of the hydroxylase (Halsey *et al.*, 2006). Because amino acids G113 and T148 are predicted to be close to the enzyme active site (Table 1), the altered outcome of propionate inactivation observed in mutant strains G113N and T148C suggests that propionate enters the active site of BMO. Although it remains unclear why mutant strains G113N and T148C display altered propionate-dependent inactivation relative to wild-type, two possibilities can be discussed. First, structural prediction programs indicate that the size of the hydrophobic substrate-binding cavity of BMO will be decreased by the G→N mutation and increased by the T→C mutation (Halsey *et al.*, 2006). In this context, the insensitivity of mutant strain G113N to propionate may indicate that the active site of the enzyme is inaccessible to propionate. In contrast, the larger substrate-binding cavity of T148C may increase the accessibility of the active site to propionate. Second, the altered outcome of propionate-dependent inactivation in the mutant strains might reflect a subtle change in the tendency of these enzymes to release H₂O₂. Indeed, recent research on a structurally related diiron desaturase indicated that a mutant form of the enzyme containing a single amino acid substitution near the active site produced 40-fold more H₂O₂ than the wild-type enzyme (Guy *et al.*, 2006). Clearly, we are in a position to move forward and obtain crystal structures of BMO from

both the wild-type and mutant strains, and carry out *in vitro* studies with the recently purified BMO (Dubbels *et al.* 2007) to gain a better understanding of short-chain fatty acid inactivation.

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