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Mutagenesis of the 'leucine gate' to explore the basis of catalytic versatility in soluble methane monooxygenase

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ABSTRACT

Soluble methane monooxygenase (sMMO) from methane-oxidizing bacteria is a multicomponent non-heme oxygenase that naturally oxidizes methane to methanol and can also co-oxidize a wide range of adventitious substrates, including mono- and di-aromatic hydrocarbons. Leucine 110, at the mouth of the active site in the α-subunit of the hydroxylase component of sMMO, has been suggested to act as a gate to control access of substrates to the active site. Previous crystallography of the wild-type sMMO has indicated at least two conformations of the enzyme that have the 'leucine gate' open to different extents and mutagenesis of homologous enzymes has indicated a role in control of substrate range and regioselectivity with aromatic substrates. By further refinement of the system for homologous expression of sMMO that we developed previously, we have been able to prepare a range of site directed mutants at position 110 in the α-subunit of sMMO. All the mutants (with Gly, Cys, Arg and Tyr, respectively, at this position) showed a relaxation of the wild-type regioselectivity with monoaromatic substrates and biphenyl, including the appearance of new products arising from hydroxylation at the 2- and 3- positions on the benzene ring. Mutants with the larger Arg and Trp at position 110 also showed a shift in regioselectivity during naphthalene hydroxylation from the 2- to the 1-position. No evidence was found, however, that mutagenesis of Leu 110 could allow very large substrates to enter the active site, since the mutants (like the wild-type) were inactive towards the triaromatic hydrocarbons anthracene and phenanthrene. Thus, our results indicate that the 'leucine gate' in sMMO is more important in controlling the precision of regioselectivity than the size of substrates that can enter the active site.

INTRODUCTION

Soluble methane monooxygenase (sMMO) is one of two enzyme systems via which methane-oxidizing bacteria catalyse the oxygenation of methane to methanol, which is the particularly challenging first step in the metabolism of the kinetically unreactive methane molecule (28). sMMO is a multicomponent enzyme encoded by the six-gene operon *mmoXYBZDC*, in which the binuclear iron active centre (8, 45) that is the site of methane oxidation resides within the α-subunit of the hydroxylase component, encoded by *mmoX* (5). It may be because methane is a small and unfunctionalized substrate that the hydrophobic pocket on the hydroxylase component of sMMO that has evolved to bind methane is also able to accommodate a very wide range of hydrocarbons and other molecules. Indeed, sMMO, whose known substrates number over 100, range in size from methane to naphthalene (2) and biphenyl (23) and also include carbon monoxide and ammonia (37), must surely be among the most catalytically versatile of any known enzyme.

The unusual catalytic versatility of sMMO has led to interest in its potential as a biocatalyst for bioremediation and synthetic chemistry, as well as an interest in how the structure of the enzyme facilitates so wide a substrate range. The hydrophobic substrate binding site of sMMO, which is believed to be responsible for binding its wide range of substrates, is adjacent to the binuclear iron active centre and is deeply buried in the 250-kDa $(\alpha\beta\gamma)_2$ hydroxylase component (9, 12, 31), presumably to prevent the solvent from quenching the highly oxidizing di-ferryl intermediate Q (22, 33, 35) that is needed to oxygenate methane and other recalcitrant substrates. Access to the active site is likely to be via another hydrophobic pocket, cavity two, which is

part of a chain of buried cavities that communicate between the active site and the solvent. Between cavity two and the active site lies the 'leucine gate', residue Leu 110. Different crystal forms of the hydroxylase differ in the conformation of Leu 110, such that in the crystal state it can either block the pathway between cavity two and the active site, or (in the alternative conformation) open a 2.6-Å diameter channel between the two cavities. A larger conformational change, such as may be caused by interaction with the other components of the sMMO complex, could open this 'leucine gate' further, to allow passage of substrates and products (30), reasonably acting to control access of substrates to the active site.

Other monooxygenases homologous to sMMO, which naturally oxidize molecules considerably larger than methane, have narrower substrate ranges than sMMO (21). There is an X-ray crystal structure for one of these enzymes, toluene *o*-xylene monooxygenase (ToMO), in which the opening to the active site is wider than that observed in sMMO (33). Whilst a more open active site is consistent with the larger natural substrate of this enzyme, it is clear that the active site of sMMO can open sufficiently to allow entry of aromatic substrates.

Mutagenesis studies of homologous monooxygenases whose natural substrates are monoaromatic hydrocarbons have indicated that the equivalent position to Leu 110 is indeed important in the interaction between these enzymes and their substrates. In a directed evolution study of toluene 2-monooxygenase (T2MO) of *Burkholderia cepacia* G4 (4), a Val to Ala mutation at position 106, equivalent to Leu 110 in sMMO, gave increased naphthalene and phenanthrene oxidation but no change in regioselectivity with naphthalene. Both mutant and wild-type gave predominantly 1-

naphthol as the product. This was consistent with the proposed gating role of this residue, i.e. the mutation affected the rate of oxidation of large substrates but did not greatly impact on regioselectivity. Similar results were found in a saturation mutagenesis study of toluene 4-monooxygenase (T4MO) where the V100L mutant showed a two-fold increase in the rate of oxidation of 2-methoxyphenol; here a modest loss of regiospecificity was observed, but the mutant (like the wild-type) gave 3-methoxyresorcinol as the major product (41). A reduction in rate of oxindole formation from indole by the Ala, Cys and Val mutants of the equivalent Ile 100 in T4MO (40) was also consistent with such a function, although a change in the predominant product of phenol hydroxylation from catechol to hydroquinone (i.e. a shift in regioselectivity from the 2- to 4-position) in the I100Q mutant of ToMO (42) indicates that the equivalent site to Leu 110 may have a role in determining regioselectivity as well as substrate access. Results from mutagenesis of the nearby site Gly 113 in soluble butane monooxygenase (sBMO) have also suggested that a conformational change involving the 'leucine gate' (Leu 109 in this enzyme) may be necessary for product release from the active site, since the G113N mutant showed a reduced inhibition by nascent methanol produced by oxidation of methane (13).

In order to probe the role of Leu 110 in sMMO, we here report further refinements in the homologous expression system for sMMO that we previously developed (25, 38) in order to overcome problems associated with expression of the enzyme in *Escherichia coli*. Several mutants at position 110 in the α-subunit of the hydroxylase have been analysed using bulky aromatic substrates in order to evaluate the role of this residue in the sMMO system and to compare its function to that of the equivalent residues in the aromatic monooxygenases.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Details of plasmids and methanotroph strains used during this study are given in Table 1. Plasmid construction and site-directed mutagenesis were accomplished using *E. coli* strains INVαF', TOP10 (Invitrogen) and XL-10 GOLD (Stratagene). The donor strain for conjugation of plasmids into *Ms. trichosporium* was *E. coli* S17-1 (36). *E. coli* strains were cultivated at 37 ºC in LB medium or on LB agar plates. Strains of *Ms. trichosporium* were cultivated at 30 ºC on nitrate mineral salts (NMS) agar plates and in NMS medium in flask and fermentor culture, using methane as the carbon and energy source, as described previously (38). As previously described, cells were cultivated in a fermentor in NMS medium containing 0.1 mg litre^{-1} (0.4 μM) of $CuSO_4.5H_2O$ and induction of sMMO occurred when the culture OD_{600nm} exceeded about 5.0, when the copper-to-biomass ratio became low enough to induce sMMO genes. Where necessary, antibiotics were added to culture media at the following working concentrations: ampicillin, 50-100 μg ml^{-1}; gentamicin, 5 μg ml^{-1}; kanamycin, 50 μg ml^{-1}.

General DNA methods

General methods for DNA purification and analysis and cloning in *E. coli* were performed according to Sambrook et al. (32). Where necessary, to facilitate difficult plasmid manipulations, commercially prepared competent *E. coli* cells (TOP10 [Invitrogen] or Solo Pack GOLD [Stratagene]) were used. DNA probes were labelled

by random priming (10, 11) using hexanucleotide primers and dNTPs from Roche according to the manufacturer's instructions. Agarose gel-purified DNA fragment (25-50 ng) was labelled with [α-^{32}P] dGTP (50 μCi) for 1 h at 37 °C with Klenow polymerase (Invitrogen). Unincorporated label was removed using MicroSpin™ columns (Amersham Pharmacia Biotech). The probes were denatured by the addition of NaOH to a final concentration of 0.4 M and incubated for 2 min at room temperature before being added to the hybridization solution.

Construction of plasmids

In order to allow subsequent manipulations of the sMMO operon using *Bam*HI, the *Bam*HI site was deleted from the vector pBBR1MCS-5 (20) by removing the fragment of the multiple cloning site that contained it with *Cla*I and *Bst*BI and religating the plasmid backbone via the compatible cohesive ends thus produced, to give the vector pBBR1MCS-5b. The 10-kb fragment containing the *mmoXYBZDC* operon from *Ms. trichosporium* OB3b and flanking sequences was excised from pTJS176 (38) with *Kpn*I and cloned into the unique *Kpn*I site of pBBR1MCS-5b, to give plasmid pMD2. The plasmid, pMD-Mdel3, to direct deletion of the chromosomal copy of the sMMO-encoding operon was then constructed as detailed in Fig. 1.

Site-directed mutagenesis and preparation of cells expressing the mutant enzymes

Site-directed mutagenesis of the *mmoX* gene, encoding the α-subunit of the sMMO hydroxylase, was performed via the four-primer overlap extension method (15) as described previously (38). Primers used for mutagenesis are given in Table 2. Dye termination sequencing was used to confirm the absence of unwanted mutations from PCR-derived portions of the cloned mutant sMMO genes. The mutant *mmoX* genes, assembled into an intact *mmoXYBZDC* operon in the sMMO expression plasmid pTJS175 (38), were introduced into *Ms. trichosporium* SMDM by conjugation (25, 38). The recombinant sMMO strains were initially grown on NMS plates containing antibiotics (gentamicin and kanamycin) and screened for sMMO activity using the naphthalene oxidation test (1, 2). Cells for more detailed analysis were prepared by growing the recombinant *Ms. trichosporium* cells in a fermentor to an OD_{600nm} greater than 5 in order to induce mutant enzyme expression from the native sMMO promoter in pTJS175, which is induced by low copper-to-biomass ratio in the culture.

sMMO assays

sMMO is the only enzyme system expressed in *Ms. trichosporium* that is able to oxygenate aromatic hydrocarbon substrates. As a result, assays with these substrates can be performed using whole sMMO-expressing cells. The semiquantitative naphthalene oxidation test, which is based on the derivatisation of the naphthol products of naphthalene oxidation to pink/purple diazo dyes, was performed on cultures in liquid medium and on agar plates as described previously (1, 2). After

oxidation of aromatic hydrocarbons by cells expressing mutant or wild-type sMMO, product analysis was performed as follows. Methanotroph cells were resuspended in 25 mM MOPS buffer (pH 7.0) to an OD_{600nm} of greater than 5. Each assay was conducted with 5 ml of cell suspension or 10 ml where biphenyl was the substrate. Oxidation substrate was added, together with sodium formate (10 mM) to ensure a plentiful supply of the reducing equivalents required by sMMO. The amount of oxidation substrate used was 50 µl for liquid substrates (toluene or ethylbenzene) and 1 mg for solid substrates (naphthalene, biphenyl, phenanthrene or anthracene). The reaction was incubated aerobically at 30 ºC for 48 h with shaking (250 rpm) in a closed 30-ml glass vial held in a horizontal position. The hydroxylated products were extracted into 1.0 ml of diethyl ether, which was then evaporated to a volume of 50 µl before analysis. The products of toluene and biphenyl oxidation were characterised by means of gas chromatography (GC), using a 6890 GC apparatus (Hewlett Packard) fitted with a Stabilwax capillary column with a Carbowax PEG coating (50 m × 0.32 mm; coating thickness, 1 µm) and coupled to a flame ionisation detector. The column temperature at the beginning of the separation was held at 100 ºC for 5 min, whereafter it was ramped to 180 ºC at 2 ºC min^{-1}, followed by 15 min at 180 ºC. The flow rate of carrier gas (nitrogen) was 1.5 ml min^{-1} and the split ratio was 10:1. When the substrate was naphthalene or ethylbenzene, products were analysed via GC-MS using a 5890 GC (Hewlett Packard) coupled to a Trio-1 mass spectrometer. Here, the GC was fitted with a Hewlett Packard HP-5 column with a (5 % phenyl) methyl polysiloxane coating (50 m x 0.32 mm; coating thickness 0.25 µm) and operated with a carrier gas (nitrogen) flow rate of 1.5 ml min^{-1}. With ethyl benzene as the substrate, the split ratio was 30:1 and the column temperature was ramped from 80ºC to 250 ºC at 4 ºC min^{-1}, followed by 1 min at 250 ºC. When naphthalene was the substrate, the

separations were carried out without split and the column temperature was ramped from 80ºC to 126 ºC at 10 ºC min^{-1}, followed by 126 ºC to 129 ºC at 0.1 ºC min^{-1} and then 129 ºC to 250 ºC at 10 ºC min^{-1}. When biphenyl was the substrate, the products were separated via a Perkin-Elmer autosystem GC using the Hewlett Packard HP-5 column (details as above) coupled to a flame ionisation detector. The separations were carried out without split. The carrier gas (nitrogen) flow rate was 1 ml min^{-1} and the column temperature was ramped from 90 ºC to 190 ºC at 5 ºC min^{-1}, held at 190 ºC for 1 min and then ramped to 280 ºC at 10 ºC min^{-1}. Products were identified by comparison of retention times and mass spectra to authentic standards.

RESULTS

Constuction of a new sMMO-deleted methanotroph expression host for recombinant sMMO enzymes

Previous mutants of the hydroxylase component of sMMO were expressed in the sMMO negative mutant of *Ms. trichosporium* known as mutant F (38). The chromosomal copy of the sMMO operon in mutant F (26) has a deletion of a 1.2 kb fragment that encompasses the portion of *mmoX* coding for amino acids Val 112-Thr 508. This limits the region of the sMMO operon that can be mutated to the portion of *mmoX* (encoding the α-subunit of the hydroxylase) that is deleted from mutant F, because mutations outside this region could be repaired by homologous recombination between the mutant plasmid-encoded sMMO operon and the remainder of the chromosomal copy. The mutagenesis target during this study, Leu 110, lies just outside this region and so it was in principle possible that mutations could be repaired by homologous recombination with the chromosome. Furthermore, for future studies we wished to produce an expression host for recombinant sMMO that would allow mutagenesis anywhere within the hydroxylase component (encoded by *mmoX*, *Y* and *Z*). The new expression host, *Ms. trichosporium* SMDM (for **s**oluble **m**ethane monooxygenase **d**eleted **m**utant), was therefore constructed, in which the first five genes of the sMMO operon (*mmoXYBZD*, including all the structural genes for the hydroxylase and protein B) had been fully deleted by marker-exchange mutagenesis (Fig. 1). The deletion of the intended portion of the sMMO-encoding operon and double recombination into the chromosome, were confirmed by restriction digestion of chromosomal DNA, digestion with *Cla*I and *Sph*I, followed by Southern blotting

and probing with probes specific to *mmoR*, *mmoX* and *mmoY* (data not shown). Strain SMDM exhibited the expected sMMO-minus phenotype, as judged by a negative naphthalene oxidation test under low-copper conditions, which induce sMMO expression and under which a parallel control with wild-type *Ms. trichosporium* OB3b showed strong naphthalene oxidation activity.

The suitability of strain SMDM as a host for expression of recombinant sMMOs was established by introducing pTJS175, containing the wild-type sMMO operon, into SMDM to give strain SMDM-sMMO (Table 1). Growth on NMS agar medium containing a low level of copper (II) (0.4 μM) to induce sMMO, yielded colonies that gave a strong positive naphthalene oxidation test, confirming expression of the recombinant sMMO.

Mutagenesis of leucine gate residue produced mutants that gave an unusual naphthalene oxidation test result

Four mutants of the proposed gating residue Leu 110 were constructed, which gave a range of amino acid bulk and functionality at this position. Mutations to Gly (less bulky; more flexible), Cys (less bulky), Arg (slightly more bulky; positively charged); Tyr (more bulky; hydroxylated) were constructed, confirmed by DNA sequencing and expressed in the *Ms. trichosporium* SMDM homologous expression system, as described in the Material and Methods. When the four mutant strains were grown in liquid culture at a low copper-to-biomass ratio, which induces expression of sMMO, all four gave positive naphthalene tests, confirming that all the mutant clones produced mutant enzymes that were active with naphthalene as the substrate. The

L110G and L110C mutants gave a positive naphthalene test where the purple diazo dye product was, like that from the wild-type, stable for more than 10 min. The colour produced by the L110R and L110Y mutants was, however, stable for less than 1 min and appeared pink compared to the purple seen with the wild-type (data not shown). Although we had previously observed mutants of sMMO where the naphthalene test gave a very weak colour change, indicating a low-activity or unstable enzyme (38), this was the first time that we had observed a mutant where the stability (and colour) of the naphthalene test result was markedly changed.

The L110R and L110Y mutants of sMMO show inverted regioselectivity with naphthalene compared to the wild-type

It was reasoned that the qualitative difference in stability of the naphthalene test colour among the mutant and wild-type enzymes was most likely due to an alteration in the position(s) of oxygenation in the products of naphthalene oxidation by the L110R and L110Y mutants. GC-MS analysis (Table 3) confirmed that there were significant alterations in the distribution of monohydroxylated products from the mutant enzymes. Whereas the wild-type enzyme and L110C mutant gave a slight excess of 2-naphthol over 1-naphthol, this regioselectivity was reversed in the other three mutants and the two mutants that gave the unstable naphthalene test colour (L110R and L110Y) yielded the smallest relative amount of 2-naphthol. Other possible products, such as dihydroxylated moieties, were not detected. A separate experiment using 10 μM solutions of the two isomers of naphthol and the same concentration of tetrazotised o-dianisidine as used in the naphthalene test for sMMO activity revealed that the diazo dye produced by 1-naphthol forms more quickly and

decays more quickly than that produced by 2-naphthol (Fig. 2). Thus the difference in regioselectivity of the mutants accounts for the visual differences in the results of their naphthalene tests.

The L110 mutants showed a relaxed regioselectivity and generated novel products with substituted monoaromatic substrates and biphenyl

With the substituted monoaromatic substrates toluene and ethylbenzene, the wild-type sMMO exhibits a mixture of side-chain hydroxylation and ring hydroxylation at the *p*-position, with ring hydroxylation predominating with toluene and sidechain hydroxylation with ethyl benzene. With toluene as the substrate, all the mutants at position 110 showed relaxed regioselectivity (Table 4), with the appearance of significant amounts of *m*-cresol, which is not seen with the wild-type. Interestingly, when Leu 110 was replaced by the smaller glycyl and cysteinyl residues, with toluene as the substrate more benzyl alcohol was produced than *p*-cresol. When ethylbenzene was the substrate, all the mutants showed a very large shift from sidechain to ring hydroxylation and at least one new product (2-ethylphenol) was observed with each mutant (Table 5). 3- and 4-ethylphenol were not resolved in these experiments, and so it is not clear whether the product 3-ethylphenol was produced. With biphenyl as the substrate, the wild-type produced predominantly 4-hydroxybiphenyl. Mutants with the larger tyrosine or arginine at position 110 produced substantially more 2-hydroxybiphenyl than the wild-type. The mutants with the smaller residues (glycine and cysteine) at this position yielded the new product 3-hydroxybiphenyl (Table 6).

Neither mutants nor wild-type oxidized triaromatic hydrocarbons

Cells expressing wild-type sMMO and all four mutants at position 110 were initially screened for oxidation of the triaromatic compounds phenanthrene and anthracene in the same manner as used for the semiquantitative naphthalene assay, by addition of tetrazotised *o*-dianisidine, which would give coloured diazo dyes after reaction with the hydroxylated products. No visible colour changes were seen from mutants or wild-type, although a control of 9-phenanthrol gave a coloured product with a detection threshold of 5 μM. GC-MS was used to analyse assay reaction that had been incubated for 30 ºC for 48 h, as described in the Material and Methods, but no hydroxylated products could be found from either of the triaromatic substrates with wild-type *Ms. trichosporium* OB3b or any of the mutant strains. Presuming that phenanthrene and anthracene are able to diffuse into the cells, these results strongly suggest that neither wild-type sMMO nor the mutants are able to oxidise these bulky aromatic substrates.

DISCUSSION

Further development of the expression and mutagenesis system

Mutagenesis of the hydroxylase component of sMMO requires the use of an especially developed expression system because attempts to date to obtain expression of active hydroxylase in *E. coli* have been unsuccessful (44). Expression of active sMMO has been reported in various heterologous systems (16, 17, 24) as well as the homologous system used here. The previous work that we performed using the homologous expression system for sMMO, which enabled the first mutagenesis of the active site of the enzyme (38), utilised a homologous expression host (*Ms. trichosporium* mutant F [26]) from which only part of the *mmoX* gene was deleted, thus limiting the region of the hydroxylase that could be mutagenised. The new strain developed here, *Ms. trichosporium* SMDM, like mutant F, is a homologous expression host that gives comparable expression of recombinant wild-type sMMO to the parental strain *Ms. trichosporium* OB3b. Moreover, strain SMDM not only allows mutagenesis anywhere within the active site-containing α-subunit, including Leu110, but also allows mutagenesis of all components of the sMMO-encoding operon except the reductase, *mmoC*, i.e. the hydroxylase (*mmoXYZ*), protein B (*mmoB*) and the *mmoD* gene, whose product may have a role in sMMO complex assembly (27).

Regioselectivity of the wild-type sMMO with aromatic substrates

Our results with *Ms. trichosporium* expressing recombinant wild-type sMMO confirmed previous results concerning the regioselectivity of sMMO with aromatic

substrates. If the substrates are considered to be roughly rectangular in shape, the wild-type enzyme shows a strong preference for hydroxylation nearest to the two short sides of the rectangle. Thus, naphthalene gives primarily 2-naphthol, biphenyl gives predominantly 4-hydroxybiphenyl and toluene and ethyl benzene undergo a mixture of side-chain and 4-position hydroxylation on the benzene ring (2, 3, 14).

Mutations at Leu 110 in sMMO relax regioselectivity but do not extend activity to triaromatic hydrocarbons

The mutant-expressing strains showed lower rates of total product accumulation than the wild-type, ranging from 2.5 to 63 % of the wild-type activities (Tables 4 and 5). Examining the data from ethyl benzene and toluene as substrate, we did not find a clear correlation between the property of the amino acid and the reduction in activity, and these results may also reflect differences in expression and stability between the mutants. Analysis of the proportions of products formed from the aromatic substrates, however, yielded information that is not biased by the level of expression.

As detailed in the Introduction, previous work on homologous monooxygenases that naturally oxidize aromatic substrates has variously indicated effects of mutations at the position equivalent to Leu 110 of the hydroxylase α subunit of sMMO on regioselectivity and substrate range. In the case of sMMO, none of the mutants, including those with substantially smaller residues at position 110, had measurable activity toward the triaromatic substrates phenanthrene and anthracene. However, all had substantial changes in regioselectivity, showing a shift away from 'terminal' oxidation of aromatic substrates, with increasing proportions of 1-naphthol from

naphthalene and 2- and 3-hydroxy products from substituted monoaromatics and biphenyl. This result was to some extent unexpected, since a "gating role" for Leu 110 suggested that mutations to smaller residues would be likely to let larger substrates into the active site. There was some correlation between the changes in product distribution and the size of the residue at position 110. The shift in regioselectivity of naphthalene oxidation was greatest in the mutants with largest residues (Tyr and Arg) at position 110. Conversely, the novel product from biphenyl oxidation, 3-hydroxybiphenyl, is seen only in the mutants with the smaller residues (Gly and Cys) at this position. The shift from side-chain to ring hydroxylation of toluene was greatest in the mutants with smaller residues (Gly and Cys) at position 110 and the corresponding shift in regioselectivity in oxygenation of ethyl benzene was almost two orders of magnitude greater than that seen with any of the other mutants. In the homologous sBMO, the mutation G113N, which is near to the 'leucine gate' (Leu 109 in butane monooxygenase), also led to a shift in regioselectivity away from the ends of the substrate molecules, in this case giving rise in regiospecificity of oxygenation of butane and propane from predominantly terminal to predominantly subterminal (13).

In monooxygenases of the naphthalene dioxygenase (NDO) family, a residue at the narrowest part of the active-site pocket (Phe 352 in NDO from *Pseudomonas* sp. NCIB 9816-4 [18]) has also been shown, via mutagenesis studies (19, 29), to influence substrate range and regioselectivity. X-ray crystallography of an enzyme-product complex indicated that the product (and presumably also the substrate) contacts Phe 352 when bound in the active site (6). Unfortunately there are currently to our knowledge no structural data for sMMO or its homologues complexed with

aromatic substrates, but the situation with Leu 110 in these enzymes seems likely to be different, since this residue is on the edge of the active site and less likely to contact the substrate at the moment of oxygenation.

Our data indicate that Leu 110 is an important residue in determining the precision of regioselectivity of sMMO with aromatic substrates. The varied mutants we have made at this position have a relaxation of the regioselectivity for aromatic substrates, which could be advantageous in activating polyaromatic pollutants for biological breakdown by consortia of microorganisms. Whilst our results do not allow us to judge whether Leu 110 functions as a substrate gate, it they strongly suggest that it is not the limiting factor on the size of aromatic substrates that can enter the active site. It may be that the important role that Leu 110 has in in determining the catalytic specificity of the enzyme may be mediated via conformational changes of the type already shown to affect this residue in various crystal forms of the enzyme (30). In future we propose to undertake detailed kinetic and structural analysis of the mutants to determine how their effect on the regioselectivity of the enzyme is mediated.

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FIGURE LEGENDS

Fig. 1. Construction of the plasmid pMD-Mdel3 and its use for marker-exchange deletion of the first five genes of the sMMO operon. In addition to the sMMO-encoding *mmoXYBZDC* operon, the upstream *mmoR* and *mmoG* genes are also indicated. These encode, respectively, a putative σ^{54}-dependent transcriptional regulator and molecular chaperon that (whilst not part of the mature sMMO complex) are needed for expression of active sMMO *in vivo* (39). PCR primers for amplification of *'mmoC* were mmodel-1 (5'-ATA ATA GGA TCC ATC GTC ATC GAG ACC GAG GAC G-3', *Bam*HI site underlined) and the M13 universal sequencing primer (3'-GTA AAA CGA CGG CCA GT-5'). Deletion of *mmoXYBZD* and the upstream region of the *mmoC* gene was effected by introducing pMD-Mdel3 into *Ms. trichosporium* by conjugation (25) and selecting exconjugants using gentamicin. *Ms. trichosporium* SMDM, the exconjugant in which the sMMO-encoded operon had been deleted via double homologous recombination, was selected on the basis of its sMMO-negative phenotype (as determined by the naphthalene oxidation test [1, 2]), together with its Gm^R Kn^S phenotype. Not drawn to scale.

Fig. 2. Stability of the coloured diazo compound formed by reaction of tetrazotised *o*-dianisidine with 1-naphthol (solid line) or 2-naphthol (dotted line).

TABLES

TABLE 1. Plasmids and *Ms. trichosporium* strains

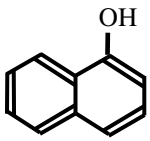
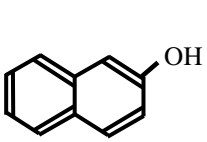
Plasmid or strain	Description	Source or reference
Plasmids		
p34S-Gm	Broad-host-range Tn*Mod*-derived plasposon, 3.6 kb; Ap^R, Gm^R	7
pBBR1MCS5	Broad host-range cloning vector, 4.8 kb, Gm^R, Mob^+, *lacZ'*	20
pBBR1MCS5b	Broad host-range cloning vector, 4.3 kb, Gm^R, Mob^+, *lacZ'*	This study
pK18mobsacB	Mobilizable plasmid with narrow host-range (pUC18-derived) replicon, 3.8 kb; *oriT*, Km^R, *lacZ'*, *sacB*	34
pUC19	Cloning vector, 2.7 kb, Ap^R, *lacZ'*	43
pMD2	10-kb *Kpn*I insert of pTJS176, containing *mmoRGXYBZDC*, cloned into pBBR1MCS5b; Gm^R	This study
pMD-Mdel1	pUC19 containing a 2.2kb fragment spanning *mmoG* of *Ms. trichosporium* and a 1.2 kb fragment containing *'mmoC*; Ap^R	This study
pMD-Mdel2	pMD-Mdel1with the 0.87-kb Gm^R cassette of p34S-Gm cloned into the *Bam*HI site between *mmoG* and *'mmoC*; Gm^R; Ap^R	This study
pMD-Mdel3	pK18mobsacB containing the 4.3-kb *mmoG*-Gm^R-*'mmoC* fragment from pMD-Mdel2; Gm^R, Kn^R	This study
pNPB101	PUC18 carrying the 1.0-kb *Xba*I-*Nde*I fragment of pSJH1a, including *mmoX'*	This study
pTJS140	Broad host-range cloning vector, 7.5 kb; Mob^+, Ap^R, Sp^R, Sm^R, *lacZ'*	38
pTJS175	10-kb *Kpn*I insert of pTJS173 cloned into pTJS140 in the same direction as *lacZ'*; Ap^R, Sp^R, Sm^R	38
pTJS176	10-kb *Kpn*I insert of pTJS173 cloned into pMTL24; Ap^R	38
Ms. trichosporium strains		
OB3b	Wild-type, sMMO-positive strain	Laboratory stock
mutant F	Kn^R, sMMO-minus double crossover mutant of OB3b partially deleted in *mmoX*	26
SMDM	Gm^R, sMMO-minus double crossover mutant of OB3b deleted in *mmoXYBZD* and the first three codons of *mmoC*	This study
SMDM-sMMO [a]	SMDM into which pTJS175 had been introduced by means of conjugation	This study

TABLE 2. Primers used for site-directed mutagenesis.

Mutation	Oligonucleotide (5'-3') [a]
L110G	GTG ATC TCG AAC TTC **GG**C GAG GTC GGC GAA TAT A Gly 110
L110C	GTG ATC TCG AAC TTC **TG**C GAG GTC GGC GAA TAT A Cys 110
L110R	GTG ATC TCG AAC TTC C**G**C GAG GTC GGC GAA TAT A Arg 110
L110Y	GTG ATC TCG AAC TTC **TAT** GAG GTC GGC GAA TAT A Tyr 110

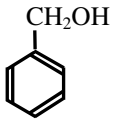
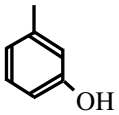
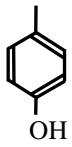
[a] Mutations are shown in bold type. Codons encoding altered amino acids are underlined. The pairs of mutagenic primers used to create each mutation were complementary and so only the forward primer is shown.

TABLE 3. Products of naphthalene oxidation by the mutants

Enzyme	Products (molar %)	
	1-naphthol 	2-naphthol 
Wild-type	43.0 ± 0.4 [a]	57.0 ± 0.4
L110G	64.0 ± 0.5	36.0 ± 0.5
L110C	46.2 ± 6.3	53.8 ± 6.3
L110Y	74.8 ± 0.7	25.2 ± 0.7
L110R	70.6 ± 6.6	29.4 ± 6.6

[a] Data are derived from three independent experiments and are shown in the form mean ± SD.

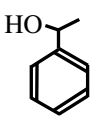
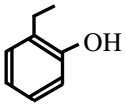
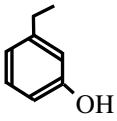
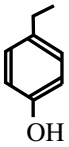
TABLE 4. Products of toluene oxidation by the mutants.

Enzyme	Products (molar %)			Molar ratio aromatic: benzylic hydroxylation	Relative total activity (%) [a]
	Benzyl alcohol 	*m*-cresol 	*p*-cresol 		
Wild-type	36.5 ± 8.7 [b]	0.0	63.5 ± 8.7	1.7	100
L110G	53.7 ± 12.5	14.6 ± 6.7	31.7 ± 9.7	0.86	12
L110C	59.4 ± 12.1	11.2 ± 7.7	29.4 ± 5.4	0.68	7.4
L110Y	34.9 ± 8.2	17.8 ± 7.4	47.3 ± 0.8	1.9	2.8
L110R	28.0 ± 6.5	14.0 ± 3.1	58.0 ± 9.7	2.6	2.5

[a] Relative total activities with toluene as the substrate, which were corrected for differences in culture OD_{600}, are given as percentages of the increase in total product concentration with wild-type *Ms. trichosporium* OB3b, which was 12 μM h^{-1}.

[b] Data are derived from three independent experiments and are shown in the form mean ± SD.

TABLE 5. Products of ethyl benzene oxidation by the mutants.

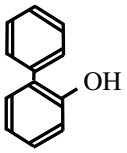
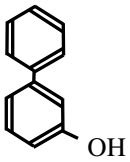
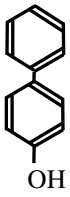
Enzyme	Products (molar %)				Molar ratio aromatic: sidechain hydroxylation	Relative total activity (%) [b]
	1-phenyl-ethanol 	2-ethyl-phenol 	3-ethyl-phenol 	4-ethyl-phenol 		
Wild-type	95.4 ± 1.4 [b]	0.0	4.6 ± 1.4 [a]		0.048	100
L110G	9.2 ± 3.1	8.8 ± 0.4	82.1 ± 2.7		9.9	32
L110C	23.6 ± 6.2	3.1 ± 2.1	73.3 ± 4.4		3.2	48
L110Y	40.2 ± 10.5	3.1 ± 0.6	56.8 ± 10.0		1.5	8.7
L110R	22.1 ± 1.6	1.3 ± 0.1	76.6 ± 1.5		3.5	63

[a] 3- and 4-ethyl phenol were not resolved in these experiments and so the mol fractions stated are the sum of both.

[b] Data are derived from three independent experiments and are shown in the form mean ± SD.

[c] Relative total activities with ethyl benzene as the substrate, which were corrected for differences in culture OD_{600}, are given as percentages of the rate of increase in total product concentrations obtained with wild-type *Ms. trichosporium* OB3b, which was 0.52 μM h^{-1}.

TABLE 6. Products of biphenyl oxidation by the mutants.

Enzyme	Products (molar %)		
	2-hydroxybiphenyl	3-hydroxybiphenyl	4-hydroxybiphenyl
			
Wild-type	12.2 ± 3.6	0.0	87.8 ± 3.6
L110G	8.4 ± 3.8	13.0 ± 1.8	78.6 ± 2.0
L110C	11.3 ± 4.2	20.3 ± 3.5	68.3 ± 0.7
L110Y	47.2 ± 0.01	0.0	52.7 ± 0.01
L110R	59.2 ± 3.2	0.0	40.8 ± 3.2