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Identification of an Amino Acid Position That Determines the Substrate Range of Integral Membrane Alkane Hydroxylases

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Selection experiments and protein engineering were used to identify an amino acid position in integral membrane alkane hydroxylases (AHs) that determines whether long-chain-length alkanes can be hydroxylated by these enzymes. First, substrate range mutants of the *Pseudomonas putida* GPo1 and *Alcanivorax borkumensis* AP1 medium-chain-length AHs were obtained by selection experiments with a specially constructed host. In all mutants able to oxidize alkanes longer than C_{13}, W55 (in the case of *P. putida* AlkB) or W58 (in the case of *A. borkumensis* AlkB1) had changed to a much less bulky amino acid, usually serine or cysteine. The corresponding position in AHs from other bacteria that oxidize alkanes longer than C_{13} is occupied by a less bulky hydrophobic residue (A, V, L, or I). Site-directed mutagenesis of this position in the *Mycobacterium tuberculosis* H37Rv AH, which oxidizes C_{10} to C_{16} alkanes, to introduce more bulky amino acids changed the substrate range in the opposite direction; L69F and L69W mutants oxidized only C_{10} and C_{11} alkanes. Subsequent selection for growth on longer alkanes restored the leucine codon. A structure model of AHs based on these results is discussed.

The alkane hydroxylases (AHs) of *Pseudomonas putida* GPo1 and other eubacteria are of great interest for biocatalytic (37) and environmental studies (35) and as prototypes of a large family of integral membrane non-heme iron oxygenases which includes desaturases and xylene monooxygenases (24). In addition, AHs occur in pathogens such as *Mycobacterium tuberculosis* and *Legionella pneumophila*, in which they have unknown roles.

The *P. putida* GPo1 AH catalyzes the hydroxylation of linear and branched aliphatic, alicyclic, and alkylaromatic compounds (7, 20, 31); oxidation of terminal alcohols to the corresponding aldehydes; demethylation of branched methyl ethers; sulfoxidation of thioethers; and epoxidation of terminal olefins (12, 13, 18, 19) and allyl alcohol derivatives (6). One of the substrate range studies was used to estimate the approximate dimensions of the substrate-binding site (31). However, our attempts to determine the three-dimensional structure of the integral membrane AH failed, and three-dimensional structures of related proteins are not available, either. Figure 1 shows a schematic topology model of *P. putida* GPo1 AlkB based on an analysis of the hydrophobicity and gene fusions with alkaline phosphatase and β-galactosidase (34). Transmembrane (TM) helices 1 and 2, 3 and 4, and 5 and 6 are likely to form pairs because the loops connecting the three helix pairs on the periplasmic side are very short. However, nothing is known about the spatial arrangement and relative angles of the TM helices or the presence or absence of kinks. AlkB contains two iron atoms that are liganded to histidine residues located in four highly conserved, short sequence motifs (26, 28). The four sequence motifs are indicated in Fig. 1 and are located near the ends of TM helices 4 and 6. Alanine scanning has shown that the eight conserved histidines in motifs A, B, and D are indeed essential for the activity of AlkB (25). The single conserved histidine in motif C (NYXEHYG) was identified as an additional potential ligand because it is conserved in all AH sequences (28, 33). In addition, the corresponding histidine in *P. putida* mt-2 xylene monooxygenase was found to be essential for activity (M. Wubbolts, personal communication). Amino acids lining the substrate-binding pocket have not been identified.

In this study, we used selection experiments to identify an amino acid position that affects the substrate range of the *P. putida* GPo1 and *Alcanivorax borkumensis* AP1 AHs. Based on these results, we carried out site-directed mutagenesis of the equivalent position in the *M. tuberculosis* H37Rv AH, which confirmed the role of the identified position.

MATERIALS AND METHODS

Strains, plasmids, and media. Strains used in this study are listed in Table 1. Luria-Bertani broth (22) and E2 medium (15) supplemented with carbon sources or antibiotics were used throughout. MT trace elements (15) were added to minimal media. *Escherichia coli* recombinants harboring plasmids were grown with appropriate antibiotics (tetracycline, 12.5 μg/ml; ampicillin, 100 μg/ml; and gentamicin, 10 μg/ml). For *Pseudomonas fluorescens* KOB2Δ1 recombinants, gentamicin was used at 100 μg/ml. For *P. putida* GPo12 recombinants, tetracycline (12.5 μg/ml) and gentamicin (25 μg/ml) were used. Recombinants were grown on solid agar E2 media with C_5 to

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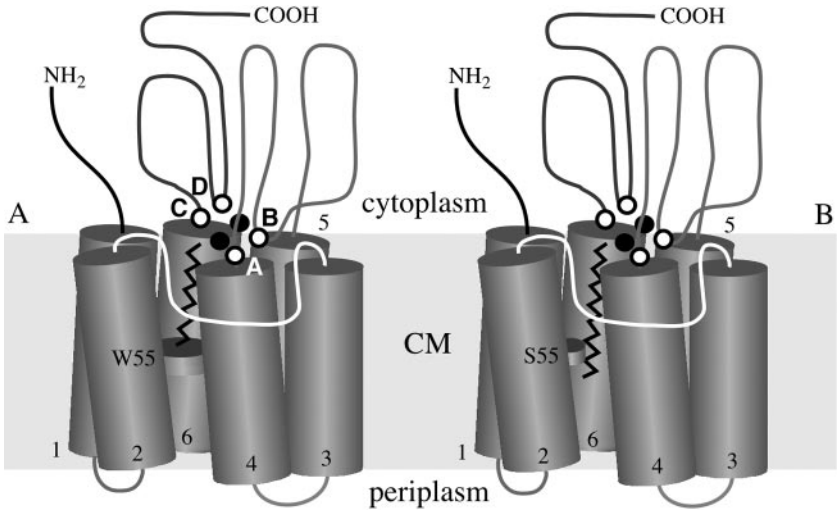

FIG. 1. Topology model of the *P. putida* GPo1 AH. The TM helices are represented by straight cylinders and numbered 1 through 6, and the connecting peptides are indicated by lines between the cylinders (not to scale). The N- and C-terminal ends of the protein are located in the cytoplasm, and the TM helices are connected by three short peptides on the periplasmic side of the membrane. About 60 to 65% of AlkB is located in the cytoplasm (34). (A) Wild-type enzyme (W55) binding dodecane; (B) W55S mutant enzyme binding hexadecane. The open circles denote the approximate locations of the four conserved histidine-containing sequence motifs (motif A, $\mathbf{H}_{138}$EXX**H**K_{143}; motif B, E_{167}**H**XXG**HH**$_{173}$; motif C, N_{269}YXE**H**YG_{275}; and motif D, L_{309}QR**H**XD**HH**A_{317}), two of which (motifs A and C) are located at the ends of TM helices 4 and 6, close to the surface of the cytoplasmic membrane. Filled circles denote the approximate locations of the two iron atoms liganded by the conserved histidine-containing sequence motifs.

C_{16} alkanes as the sole C and energy source, as described previously (27).

To determine the growth rates of *Pseudomonas* recombinants reported in Table 2 and/or to select for mutations in the *alkB* genes that would allow growth, 5-ml Luria-Bertani broth precultures with appropriate antibiotics were inoculated with single colonies resulting from electroporation or triparental matings of plasmids containing wild-type or mutant *alkB* genes.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference(s), source, or source strain
E. coli GEc137	*E. coli* DH1 *fadR*	5
E. coli DH10B	Cloning strain	Invitrogen
M. tuberculosis H37Rv	Virulent strain, possibly degrades alkanes	ATCC 27294
P. fluorescens CHA0	C_{10} to C_{28} *n*-alkane-degrading biocontrol strain	9
P. fluorescens KOB2Δ1	*P. fluorescens* CHA0 *alkB* knockout	27
P. putida GPo1	C_5 to C_{12} *n*-alkane-degrading strain	23, 33
P. putida GPo12	*P. putida* GPo1 cured of OCT	14
pCom8	Expression vector, P*alkB* Gm^r *oriT alkS*	29
pKKPalk	Expression vector, P*alkB* Ap^r	29
RK600	Helper plasmid for triparental matings	3
pGEc47ΔB	ΔB derivative of pGEc47	34
pCom8-*alkB*-GPo1	pCom8 with *alkB* from *P. putida* GPo1	27
pCom8-*alkB*-GPo1-H273A	pCom8-*alkB*-GPo1 with H273A mutation	This study
pCom8-*alkB*-GPo1-H273Q	pCom8-*alkB*-GPo1 with H273Q mutation	This study
pCom8-*alkB*-GPo1-W55S	pCom-*alkB*-GPo1 with W55S mutation	This study
pCom8-*alkB*-GPo1-W55C	pCom-*alkB*-GPo1 with W55C mutation	This study
pKKPalk-*alkB*-GPo1	pKKPalk with *alkB* from *P. putida* GPo1	This study
pKKPalk-*alkB*-GPo1-W55S	pKKPalk-*alkB*-GPo1 with W55S mutation	This study
pKKPalk-*alkB*-GPo1-W55C	pKKPalk-*alkB*-GPo1 with W55C mutation	This study
pCom8-*alkB1*-AP1	pCom8 with *alkB1* from *A. borkumensis* AP1	27, 32
pCom8-*alkB1*-AP1-W58S	pCom8-*alkB1*-AP1 with W55S mutation	This study
pCom8-*alkB1*-AP1-W58C	pCom8-*alkB1*-AP1 with W55C mutation	This study
pCom8-*alkB1*-AP1-W58L	pCom8-*alkB1*-AP1 with W55L mutation	This study
pCom8-*alkB1*-AP1-W58G	pCom8-*alkB1*-AP1 with W55G mutation	This study
pCom8-*alkB*-H37Rv	pCom8 with *alkB* from *M. tuberculosis* H37Rv	27
pCom8-*alkB*-H37Rv-L69F	pCom8-*alkB*-H37Rv with L69F mutation	This study
pCom8-*alkB*-H37Rv-L69W	pCom8-*alkB*-H37Rv with L69W mutation	This study

TABLE 2. Growth rates of *P. fluorescens* CHA0, *P. fluorescens* KOB2Δ1, *P. putida* GPo1, *P. putida* GPo12(pGEc47ΔB), and derived recombinants on *n*-alkanes ranging from C_6 (hextane) to C_{16} (hexadecane)

Strain or plasmid[c]	Growth rate (h^{-1}) on[a]:							
	C_6	C_8	C_{10}[c]	C_{11}[c]	C_{12}	C_{13}	C_{14}	C_{16}
GPo1	0.49	0.31	0.20	0.094	0.063	0.018	0	0
GPo12(pCom8-*alkB*-GPo1)	0.41	0.32	0.21	0.087	0.056	0.017	0	0
GPo12(pCom8-*alkB*-GPo1-W55S)	0.38	0.28	0.20	0.11	0.092	0.047	0	0
GPo12(pCom8-*alkB*-GPo1-W55C)	0.35	0.27	0.21	0.12	0.090	0.046	0	0
GPo12(pCom8-*alkB1*-AP1)		0.28	0.25	0.081	0.0087	0	0	
CHA0[b]					0.019		0.019	0.053
KOB2Δ1(pCom8-*alkB*-GPo1)			0.009	0.013	0.0069	0.0020	0	0
KOB2Δ1(pCom8-*alkB*-GPo1-W55S)			0.0083	0.012	0.0068	0.0075	0.011	0.012
KOB2Δ1(pCom8-*alkB*-GPo1-W55C)			0.0087	0.012	0.0070	0.0077	0.012	0.013
KOB2Δ1(pCom8-*alkB1*-AP1)			0	0	0	0	0	0
KOB2Δ1*(pCom8-*alkB1*-AP1)			0.028	0.021	0.026	0.0091	0	0
KOB2Δ1*(pCom8-*alkB1*-AP1-W58S)			0.018	0.023	0.027	0.017	0.035	0.017
KOB2Δ1*(pCom8-*alkB1*-AP1-W58L)			0.014	0.024	0.022	0.0076	0.0051	0
KOB2Δ1(pCom8-*alkB*-H37Rv)			0.025	0.027	0.043	0.043	0.041	0.038
KOB2Δ1(pCom8-*alkB*-H37Rv-L69F)			0.024	0.0081	0	0	0	0
KOB2Δ1(pCom8-*alkB*-H37Rv-L69W)			0.011	0.0053	0	0	0	0

[a] Average growth rates of two parallel cultures inoculated from the same preculture (in all cases, the difference in growth rate was less than 10%). To reduce the toxicity of C_{10} and C_{11} to KOB2Δ1 recombinants, these alkanes were diluted in heptamethylnonane (1:3 for C_{10} and 1:1 for C_{11}).
[b] Data are from reference 27.
[c] *, uncharacterized mutation in KOB2Δ1 that allows functional expression of AP1 AlkB1.

Main cultures (500-ml baffled Erlenmeyer flasks with 100 ml of E2 medium and 0.5% [vol/vol] *n*-alkanes as a carbon source) were inoculated with 1 ml of the precultures and incubated with shaking at 130 rpm and at 30°C. Citrate (0.02%) was added to *P. fluorescens* cultures. The cell densities in the cultures were estimated from the optical density at 450 nm (36).

Cultures that did not start to grow within a week were further incubated with shaking at 130 rpm and at 30°C for as long as was necessary to obtain growth (several weeks to several months). Once growth started, the optical density at 450 nm was monitored until the stationary phase, 1 ml from the cultures was used to inoculate new cultures, which were grown to the stationary phase, and plasmid was isolated and sequenced to identify mutations in the *alkB* gene. All cultures were carried out at least in duplicate.

DNA manipulations. *E. coli* strains were transformed by electroporation according to the method of Dower et al. (4). *P. fluorescens* KOB2Δ1 was transformed by electroporation according to the method of Højberg et al. (11). Plasmids were transferred to *P. putida* GPo12(pGEc47ΔB) by triparental matings (27). Plasmid DNA was isolated with a Roche highly pure plasmid isolation kit or according to the method of Birnboim and Doly (2) for *Pseudomonas* recombinants. The *P. putida* GPo1 *alkB* gene was amplified from pGEc47 (5) with primers B5-Eco (GGAGAATTCCAAATGCT TGAG [EcoRI site is underlined]) and B3-Hind (TTTGTG AAAGCTTTCAACGCC [HindIII site is underlined]) and was cloned between the EcoRI and HindIII sites of pCom8 (29). The *M. tuberculosis* H37Rv and *A. borkumensis* AP1 *alkB* genes were cloned in pCom8 as described previously (27). Mutations were introduced in the H37Rv *alkB* gene by using the QuikChange method and the primers MT-L69F-fw (TCG GACCGATCCTGTTCTACGTaTTGTTGCCGCTTCT) and MT-L69W-fw (CTGTGGATCGGACCaATCCTGtggTACGTC TTGTTGCC). The primers introduce a SnaBI site and a BstXI site (underlined), respectively, to facilitate the screening of the mutants. H273A and H273Q mutations were introduced in the GPo1 *alkB* gene by using primers GPo1-H273A-fw (GCGAACTATATTGAAgcTTACGGCTTGCTCCGT) and GPo1-H273Q-fw (ACTATATTGAACAGTACGGtcTaCTCCG TCAAAAA). Lowercase letters indicate changed bases. These primers also introduce HindIII and AccI sites (underlined). Both strands of the pCom8 inserts were sequenced on a Li-Cor 4000L sequencer with the Amersham Thermosequenase cycle sequencing kit and IRD800-labeled PalkFw3 (GCCAGCTCGTGTTTT TCCAGCAGACG) and pKKRev (GAGTTCGGCATGGGGT CAGGTG) (MWG-Biotech). Nucleotide and amino acid sequences were analyzed and compared using LASERGENE Navigator software from DNASTAR. Nucleotide and amino acid sequences were compared with the EMBL, Swiss-Prot, and GenBank databases by using the BLAST application (1). BLAST searches were carried out at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Selection for the hydroxylation of long-chain-length *n*-alkanes by the *P. putida* GPo1 and *A. borkumensis* AP1 AHs. Growth experiments using baffled Erlenmeyer flasks showed that *P. putida* GPo1 is able to grow well on alkanes ranging from C_6 to C_{13}, with growth rates ranging from 0.49 h^{-1} (doubling time, 1.7 h) for C_6 to 0.018 h^{-1} (doubling time, 40 h) for C_{13} (Table 2). Selection experiments to obtain mutants of *P. putida* GPo1 able to grow on alkanes longer than C_{13} failed also in the presence of a gratuitous inducer of the *alk* genes, dicyclopropylketone (8), and/or biosurfactants to facilitate alkane uptake by the strain, such as rhamnolipids (0.01%) or Triton X-100 (0.1%), failed to facilitate alkane uptake by this strain. This result indicates that the host *P. putida* does not allow selection for mutants that are able to oxidize longer

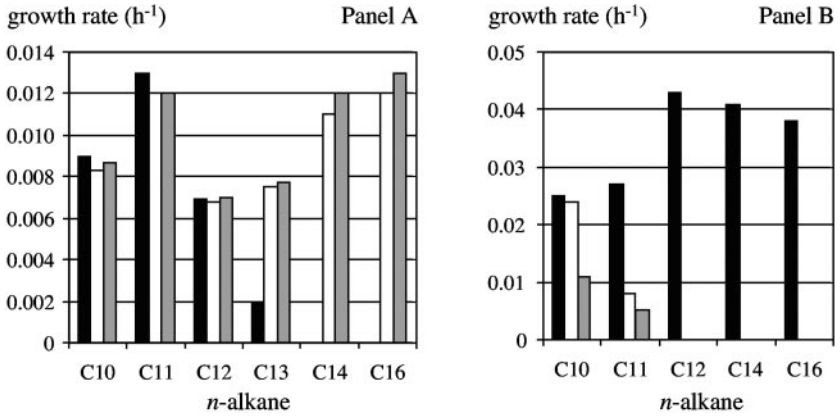

FIG. 2. (A) Growth rates on alkanes of *P. fluorescens* KOB2Δ1 containing the *P. putida* GPo1 *alkB* wild-type gene (black bars) or the *alkB* W55S and W55C mutant genes (white and grey bars, respectively). (B) Growth rates of *P. fluorescens* KOB2Δ1 containing the *M. tuberculosis* H37Rv *alkB* wild-type gene (black bars) and the L69F and/or L69W mutant gene (white bars and grey bars, respectively).

alkanes, presumably because an uptake system for such alkanes is lacking in this strain. Therefore, the *P. putida* GPo1 *alkB* gene was expressed in *P. fluorescens* KOB2Δ1, an *alkB1* knockout derivative of *P. fluorescens* CHA0 that is able to take up alkanes up to C_{28} but is unable to grow on C_{10} to C_{16} alkanes unless an AH that is able to oxidize these alkanes is expressed in this strain (27). To this end, the GPo1 *alkB* gene encoding the integral membrane monooxygenase component of the AH system was cloned in the broad-host-range expression vector pCom8 (29) and transferred to *P. fluorescens* KOB2Δ1. For details on plasmids and host strains, see Table 1.

Recombinant strain KOB2Δ1(pCom8-*alkB*-GPo1) was tested for growth on alkanes in liquid cultures, and C_{10} to C_{13} alkanes were found to support the growth of the recombinant. C_{14} and C_{16} did not support growth (Table 2; Fig. 2A). However, after 3 to 4 weeks, the latter cultures did start to grow. New cultures inoculated with these long-term cultures started to grow immediately. To investigate whether mutations in the *alkB* gene had occurred, the inserts of plasmids isolated from seven independent C_{14} and C_{16} cultures were sequenced, which revealed point mutations in all cases in tryptophan codon W55, which had changed to a serine (TGG→TCG) or cysteine (TGG→TGC or TGT) codon. The fact that three different codon changes were found indicates that these mutants are not siblings that were already present in the precultures that were used to inoculate the long-term cultures. Mutations in other positions were not found. Two mutated plasmids, pCom8-*alkB*-GPo1-W55S and pCom8-*alkB*-GPo1-W55C, were retransferred to KOB2Δ1. The resulting recombinants were able to grow on C_{14} and C_{16} immediately, while the growth rate of those on C_{13} had tripled (Table 2; Fig. 2A).

Similar selection experiments with *P. fluorescens* KOB2Δ1 were also carried out with *A. borkumensis* AP1 AlkB1, which oxidizes C_5 to C_{12} alkanes. In this case, selection of mutants was not possible with the original strain, because *A. borkumensis* AP1 contains multiple AHs with overlapping substrate ranges (32). Here, we observed that the KOB2Δ1 recombinant containing plasmid pCom8-*alkB1*-AP1 initially did not grow on any of the alkanes. Cultures with C_{14} and C_{16} as C sources did not start to grow, even after several months. However, KOB2Δ1(pCom8-*alkB1*-AP1) cultures started to grow on C_{10} and C_{12}, with delays of 1 to 2 months. Sequencing of the AP1 *alkB1* gene from the C_{10} and C_{12} cultures revealed no changes. Retransfer of the plasmids to KOB2Δ1 yielded recombinants that, again, did not grow on alkanes for 1 to 2 months, suggesting that one or more mutations in the host were required to obtain functional expression of AP1 *alkB1*. These recombinants were not further investigated. KOB2Δ1(pCom8-*alkB1*-AP1) cells from the above-mentioned C_{10} and C_{12} cultures [now named KOB2Δ1*(pCom8-*alkB1*-AP1)] did start to grow on C_{13}, C_{14}, and C_{16} after 2 to 3 weeks, and sequencing of eight *alkB1* genes from these cultures showed that tryptophan codon W58 (corresponding to W55 in GPo1 AlkB) had changed to serine (three cases), cysteine (three cases), glycine (one case), and leucine (one case). Recombinants containing W58S, W58C, and W58G mutations grew on C_{10} to C_{16} alkanes, while the W58L mutant allowed relatively weak growth on alkanes up to C_{14} but no growth on C_{16} (Table 2). Here, the growth rates of the recombinants could not be compared with those of the wild-type strain *A. borkumensis* AP1 because of the strong wall growth and clumping of this strain (32).

Effects of the W55S and W55C mutations on the growth of *P. putida* GPo12 recombinants on alkanes. To investigate (i) why we failed to obtain substrate range mutants of *P. putida* GPo1, (ii) if the activity on short- and medium-chain-length alkanes remains the same, and (iii) whether the substrate range changes upon the mutation of codon W55, two mutant GPo1 *alkB* genes (a W55S mutant and a W55C mutant) and the wild-type *alkB* gene, all in pCom8, were transferred to *P. putida* GPo12(pGEc47ΔB). This recombinant is a derivative of GPo1 in which the OCT plasmid (carrying all the *alk* genes) is replaced by the pGEc47ΔB plasmid (carrying all the *alk* genes except *alkB*) (27). In liquid cultures, *P. putida* GPo12(pGEc47ΔB) recombinants containing pCom8-GPo1-*alkB*-W55S or pCom8-GPo1-*alkB*-W55C showed growth rates on C_6 to C_{10} similar to, and growth rates on C_{11} to C_{13} higher than, those obtained with the wild-type *alkB* gene (Table 2). However, recombinants containing pCom8-GPo1-*alkB*-W55S or pCom8-GPo1-*alkB*-W55C did not grow on C_{14} or C_{16}, even after induction with dicyclopropylketone and in the presence of 0.01% rhamnolipids or Triton X-100. In combination with the observation that we were not able to obtain substrate range mu-

tants with GPo1, this result indicates that GPo1 and derived recombinants lack an uptake system for alkanes longer than C_{13}.

Mutagenesis of the *M. tuberculosis* H37Rv AH. To test the hypothesis that the position corresponding to W55 in AlkB-GPo1 affects the substrate range in long-chain-length AHs as well as in medium-chain-length AHs, we carried out site-directed mutagenesis of this position of the *M. tuberculosis* H37Rv AH, which was previously shown to oxidize C_{10} to C_{16} alkanes in KOB2Δ1 (27). Two mutants (the L69F and L69W mutants) were constructed and transferred to KOB2Δ1. The resulting recombinants grew on C_{10} and C_{11} but failed to grow on C_{12} to C_{16} alkanes, unlike the recombinant containing the wild-type sequence (Table 2; Fig. 2B). L69F and L69W mutant cultures started to grow on C_{12} to C_{16} alkanes after 2 to 3 weeks due to mutations changing the F69 and W69 codons to leucine (six cases) or serine (one case) codons.

As the L69F and L69W mutations limit the length of the accepted alkane to C_{11} and not C_{13}, as was the case with wild-type GPo1 AlkB, the substrate range of the H37Rv AlkB mutants was within the range that can be tested well in *P. putida*. In this host, we found that growth rates of recombinants containing the wild-type sequence and the L69F mutants were identical on C_9; the L69F mutant grew on C_{10} slightly slower, while the L69F recombinant initially did not grow on C_{12} to C_{13}. In all cases, cultures eventually started to grow, and the L69F mutant reverted to L69 (three mutants were sequenced).

Mutagenesis of position H273 in the *P. putida* GPo1 AH. H273A and H273Q mutant proteins of the *P. putida* GPo1 AH were constructed to test whether H273 in conserved histidine motif C is essential for activity. *P. putida* GPo12(pGEc47ΔB) recombinants containing pCom8-GPo1-*alkB*-H273A or -H273Q failed to grow on any of the alkanes tested, although the protein was expressed to normal levels (data not shown).

DISCUSSION

Medium- and long-chain-length AHs have been previously cloned and functionally expressed from several sources, including *A. borkumensis* AP1, a marine hydrocarbonoclastic γ-proteobacterium that makes up a large part of the biomass (up to 30%) in oil-polluted marine environments (10); *M. tuberculosis* H37Rv, the causative agent of tuberculosis; and *P. putida* GPo1, which expresses the prototype integral membrane alkane hydroxylase and is a useful biocatalyst (5, 17, 27, 32). These studies have allowed us to start investigating structure-function relationships. However, we have found that sequence alignments alone do not enable us to identify positions involved in substrate binding; other methods based on the selection of mutants with different substrate ranges must be applied. In this work, we show that point mutations at position W55 in *P. putida* GPo1 AlkB or at position W58 in *A. borkumensis* AP1 AlkB1 obtained by such selection experiments are sufficient to allow medium-chain-length AHs to oxidize alkanes longer than C_{13}. Because *P. putida* GPo1 appears to lack an uptake system for such alkanes (27) and *A. borkumensis* AP1 contains multiple AHs (32), the mutations could be obtained only by transferring the medium-chain-length AHs to a host that is able to take up long-chain-length alkanes but no longer possesses an AH that oxidizes these substrates: *P. fluorescens* KOB2Δ1 (27).

Close to 40 full-length AH gene sequences are now available, many of which have now been shown to encode functional AHs (17, 21, 27, 30). With a few exceptions, the encoded protein sequences show more than 40% sequence identity to each other. All contain the six hydrophobic stretches proposed to be TM helices in GPo1 AlkB, the level of sequence identity being somewhat lower than the average in these stretches. Residue W55 of the GPo1 AH is located close to the middle of TM helix 2. Figure 3 shows a multiple sequence alignment for this helix. In the highly homologous medium-chain-length AHs of *P. putida* P1 (28, 33) and AlkB1 of *A. borkumensis* AP1 (32), the corresponding position is also occupied by a tryptophan residue. In contrast, the other AHs have an alanine, a valine, a leucine, or an isoleucine residue at the corresponding position. Eleven of these other AHs were shown to oxidize alkanes that had up to at least 16 carbon atoms (17, 21, 27, 30) (Fig. 3). Not one of these enzymes was limited to the medium-chain-length alkanes.

The length of a C_{13} molecule in an extended (linear) conformation is about 19 Å. Assuming that the six TM segments of integral membrane AHs fold as perfect α-helices, with a pitch of 1.5 Å per residue, a C_{13} molecule corresponds to 12 to 13 amino acids in an α-helix. Comparisons of the TM stretches indicate that W55 is located about 11 residues from the cytoplasmic (C-terminal) end of the hydrophobic core sequence or about 10 and 14 residues from the conserved histidines at the ends of TM helices 4 and 6, respectively, approximately the correct distance to position the activated oxygen near the end of an extended C_{13} molecule. Therefore, the W55 residue may limit the lengths of the substrate alkanes, as shown schematically in Fig. 1. More evidence for this notion was obtained by changing residue L69 of the *M. tuberculosis* H37Rv AH, which corresponds to W55 in GPo1 AlkB, to F or W. The resulting mutants still oxidized C_{10} and C_{11} alkanes but no longer oxidized C_{12} to C_{16} alkanes, confirming the importance of this position. In almost all integral membrane AHs that have not been characterized with respect to substrate range, the position corresponding to W55 in the *P. putida* GPo1 AH is a small residue, which suggests that, by far, most members of this enzyme family act on alkanes longer than those with 12 carbon atoms. The *P. putida* AHs and their close homologs from other closely related γ-proteobacteria, such as *A. borkumensis*, are exceptions in this enzyme family in that they have high activity with C_5 to C_{10} alkanes and do not oxidize alkanes longer than those with 12 or 13 carbon atoms.

To confirm that the conserved histidine in sequence motif C, at the end of TM helix 6, may play a role in binding the two iron atoms in AlkB or in catalysis, we replaced this residue with alanine and glutamine. Both mutations resulted in a complete loss of activity, suggesting that H273 is essential, like the eight histidines studied previously by Shanklin and Whittle (25).

The model shown in Fig. 1 implies that alkanes interact with the halves of the TM helices closest to the cytoplasm. The C-terminal half of TM helix 2 may be involved because the position corresponding to W55 in the AlkB protein of GPo1 is located in this TM helix, while the C-terminal halves of TM helices 4 and 6 may interact with the substrate, as two of the conserved histidine motifs are located at the ends of these two TM helices. TM helices 1 and 2 of the integral membrane AHs are absent in desaturases, enzymes that are distantly related to AHs (26). Recent studies with desaturase chimeras of the Δ^6

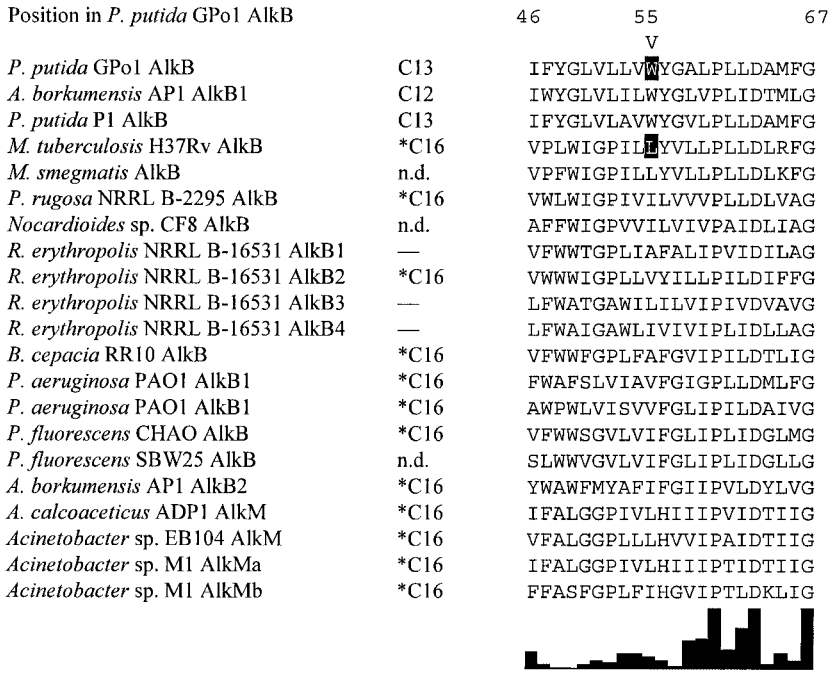

FIG. 3. Sequence alignment of TM helix 2 and comparison of properties. The first column contains the strain and AH names. *M. smegmatis*, *Mycobacterium smegmatis; P. rugosa*, *Prauserella rugosa*; *R. erythropolis*, *Rhodococcus erythropolis*; *B. cepacia*, *Burkholderia cepacia*; *A. calcoaceticus*, *Acinetobacter calcoaceticus*. The second column contains the upper end of the respective AH substrate ranges. Asterisks indicate that C_{16} was the longest alkane tested, but it is likely that the substrate range extends to longer alkanes. n.d., not determined; —, no activity could be detected. The third column shows the alignment of TM helix 2, with the position of the first and last residues indicated above (GPo1 AlkB position). TM helix 2 runs from the periplasm to the cytoplasm. Position 55 (GPo1 AlkB position) is indicated by the letter V. The W55 residue in the GPo1 AH and the L69 residue in the *M. tuberculosis* H37Rv AH are framed in black. The degree of conservation within TM helix 2 is shown below as a bar graph created by Clustal X.

fatty acid and Δ^8 sphingolipid desaturases from *Borago officinalis* indicate that TM helices 1 and 2 of these enzymes, which correspond to TM helices 3 and 4 in the AHs, form at least part of the substrate-binding site of these enzymes (16). Therefore, TM helices 3 and 4 in the AHs may play a similar role. Further site-directed mutagenesis and selection experiments will be used to clarify this question.

With these results, we have for the first time obtained insight into the substrate-binding pocket of the integral membrane AHs, enzymes that appear to be crucial in natural crude oil degradation, which is interesting for biocatalytic applications, but that also play an unknown role in pathogens such as *M. tuberculosis* and *L. pneumophila*.

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