Difference in kinetic behaviour of catechol 2,3-dioxygenase variants from a polluted environment

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In a previous environmental survey of a polluted area, the authors identified two catechol 2,3-dioxygenase (C23O) sequences predominant in environmental bacterial isolates mineralizing benzene and/or toluene and also in soil DNA extracts. In the present study, using information of stable operon arrangement and conserved gene sequences, the complete C23O ORFs of these two variants were cloned, sequenced and overexpressed. The variants differ in six nucleotide positions, and the putative protein sequences differ only by a single amino acid, Tyr or His, at position 218. Even though the three-dimensional model does not suggest a significant influence of such an amino acid substitution on enzyme function, the Tyr218 variant differed significantly from the His218 variant in lower turnover number and in lower apparent K_m for catecholic substrates. These results are evidence of the importance for enzyme function of amino acids not directly influencing active site structure and prove the utility of recovering polymorphisms evolved and selected for special functions in natural ecosystems.

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INTRODUCTION

Catechol and its substituted derivatives are common intermediates in aerobic degradation pathways of numerous natural and xenobiotic aromatic pollutants (Harwood & Parales, 1996; Reineke & Knackmuss, 1988; Smith, 1990). These compounds can be subject to intradiol or extradiol cleavage reactions, which finally lead to mineralization. Extradiol ring-cleavage by catechol 2,3-dioxygenase (C23O) is the predominant reaction in the degradation of methylcatechols (Smith, 1990), and examination of evolutionary relationships (Eltis & Bolin, 1996) has shown that the majority of C23Os are phylogenetically closely related, constituting the I.2 extradiol dioxygenase family. Subfamily I.2.A seems to be of particular importance for the degradation of monocyclic aromatic compounds (Eltis & Bolin, 1996). Various mechanistic studies have been performed (Bugg & Lin, 2001; Shu *et al.*, 1995), and the structure of an archetypal C23O from *Pseudomonas putida* mt-2 (Nakai *et al.*, 1983) has been elucidated (Kita *et al.*, 1999). It is well known that the catalytic properties of catabolic enzymes can be dramatically influenced by single amino acid changes (Beil *et al.*, 1998; Parales *et al.*, 1999, 2000; Pollmann *et al.*, 2003). Also, in the case of members

of the C23O subfamily I.2.A, significant differences in kinetic properties have been observed in variants exhibiting few amino acid differences (Kitayama *et al.*, 1996; Williams *et al.*, 1990).

Usually, studies of regions affecting enzyme catalytic parameters have been performed by the comparison of isolates expressing different C23Os (Cerdan *et al.*, 1995; Kitayama *et al.*, 1996), by the generation of chimeric proteins (Kikuchi *et al.*, 1999; Kitayama *et al.*, 1996), and/or by the selection under laboratory conditions of mutant proteins with new kinetic parameters (Cerdan *et al.*, 1994, 1995; Kikuchi *et al.*, 1999; Wasserfallen *et al.*, 1991). One alternative approach to identify amino acids important to fine-tune catalytic activities is by recovering C23O natural diversity. In previous environmental studies we identified the predominant C23O gene polymorphisms in BTEX-contaminated environments (Junca & Pieper, 2003, 2004). Samples heavily contaminated with benzene and toluene contained an abundant C23O gene fragment (527 bp in length), identical in sequence to the C23O gene of *Pseudomonas stutzeri* AN10, and which differed from the gene fragment predominant in other environments by a single base (encoding a tyrosine instead of histidine at position 218), indicating that specific C23O genes and operons have been positively selected during the adaptation of soils to BTEX contamination. We also observed that out of 19 isolates harbouring such C23O enzymes, only two isolates harbouring the His218 variant

Abbreviation: C23O, catechol 2,3-dioxygenase.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this paper are AJ544924 and AJ544930.

could grow on benzene, toluene and ethylbenzene, whereas 17 isolates containing the Tyr218 variant could grow on benzene only. Moreover, whereas the His218 variant showed the highest activity with catechol of the substrates tested, the Tyr218 variant exhibited the highest activity with 4-methylcatechol and a relatively elevated activity with 3-methylcatechol (Junca & Pieper, 2004). In two representative isolates, it could be shown that both the His218 and the Tyr218 variant are constitutively expressed and constitute the only catechol ring-cleavage enzyme observed in cell extracts during growth on benzene. In this work, we report the cloning, heterologous expression and kinetic characterization of these two C23O enzymes, which differ by a single amino acid position that alters enzyme function. This effect is analysed and discussed in the light of its structural and ecological significance.

METHODS

Strains and culture conditions. *Pseudomonas* sp. strain 1YB2, capable of mineralizing benzene, *Pseudomonas* sp. strain 1YdBTEX2, capable of mineralizing benzene, toluene and ethylbenzene, and *Escherichia coli* JM109 were cultured as previously described (Junca & Pieper, 2003; Sambrook *et al.*, 1989).

PCR and molecular biology techniques. A primer set (FER2, 5'-GCC YTG GCC TGY CRA STG TWT-3' and MUCDOCB, 5'-TTC CAG GTC ATS AGC AGY AGC GG-3') was designed based on conserved regions of C23O operon neighbouring genes (see Results). To prepare the DNA template, colonies of *Pseudomonas* sp. 1YB2, *Pseudomonas* sp. 1YdBTEX2 or recombinant *E. coli* strains were dissolved in 50 µl water, boiled for 10 min (Kanakaraj *et al.*, 1998), centrifuged, and 4 µl supernatant was used as template for PCR reactions (50 µl) containing a final concentration of 1 × PCR Buffer (Promega), 1.5 mM MgCl₂, 200 µM each deoxyribonucleotidetriphosphate, 0.25 µM each primer (synthesized by Invitrogen) and 0.3 U µl⁻¹ *Taq* DNA polymerase (Promega). The PCR programme was as follows: one step at 94 °C for 5 min, 10 cycles of touchdown PCR of 94 °C for 1 min, 63 °C (−1.5 °C per cycle) for 1 min and 72 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2.5 min, followed by final elongation at 72 °C for 8 min. To determine the correct size of the amplification fragments, the PCR products were run in 1.0% agarose gels (10 cm length, 1 × TAE running buffer, 1.0 h at 95 V) and bands visualized by ethidium bromide staining (Sambrook *et al.*, 1989). The PCR products were cleaned with the Qiaquick PCR Cleaning Kit (Qiagen) and cloned in the pGEM-T system (Promega). The ligation products were transformed in *E. coli* JM109 competent cells cultured according to the manufacturer's instructions (Promega). Colonies were screened for expression of C23O by spraying with catechol (Junca & Pieper, 2004). C23O-expressing clones were purified, and inserts from plasmids termed pC23OHis218 and pC23OTyr218 were completely sequenced on both strands using the BigDye v1.1 system in an ABI 373A automatic DNA sequencer (Perkin-Elmer Applied Biosystems) with M13 forward and reverse primers (Sambrook *et al.*, 1989) and C23O inner primers described elsewhere (Junca & Pieper, 2004). The nucleotide sequences determined in this paper can be accessed from the upgraded GenBank/EMBL/DDJB accession numbers AJ544924 and AJ544930.

Overexpression, quantification and kinetic characterization of C23Os. For overexpression of C23O enzymes, *E. coli* JM109 cells expressing C23O were grown and cell extracts prepared as described previously (McKay *et al.*, 2003). Total protein concentrations in cell

extracts were determined by the Bradford protein assay (Bradford, 1976) with BSA as standard. For quantification of C23O proteins in cell extracts, SDS-PAGE was performed on a Bio-Rad Miniprotein II, essentially as described by Laemmli (1970), with acrylamide concentrations for concentrating and separating gels of 5 and 10% (w/v), respectively. Dilutions were designed so that the amount of protein loaded ranged from 0.3 to 15 µg. Gels were incubated in a fixative solution of 10% acetic acid/40% ethanol for 30 min, washed twice for 30 min with 20% ethanol and stained overnight in 0.4 µM ruthenium II tris (bathophenanthroline disulfonate) (Lamanda *et al.*, 2004; Rabilloud *et al.*, 2001). Gels were washed in 20% ethanol/10% acetic acid to eliminate residual matrix background staining and scanned using a Fujifilm LAS-1000 CCD camera. The fluorescence intensity was integrated, and the relative intensities of the bands corresponding to the C23O protein were determined using the AIDA 2.1 software package (Raytest Isotopenmessgeräte) (Lamanda *et al.*, 2004).

N-terminal sequencing and MALDI-TOF analysis were performed as previously described (Junca & Pieper, 2004).

Catalytic activities of C23O proteins were recorded on a UV 2100 spectrophotometer (Shimadzu Corporation). Activities were determined at 25 °C in 50 mM air-saturated K/Na-phosphate (pH 7.5) with catechol, 3-methylcatechol, 4-methylcatechol or 4-chlorocatechol as substrates, using the extinction coefficients of reaction products previously described (Heiss *et al.*, 1995; Hirose *et al.*, 1994). One unit of enzyme activity was defined as the amount of enzyme that forms 1 µmol product per minute. Specific activities are expressed as units per gram of protein. C23O proteins utilize a compulsory-order ternary complex mechanism in which binding of the catecholic substrate precedes that of oxygen. Kinetic data were recorded in experiments where only one of the substrates was varied. $V_{\max}$, k_{cat} and apparent K_{mA} values ($K_{\text{mA}}^{\text{app}}$) for catecholic substrates were determined using 1–100 µM substrate in air-saturated buffer, and kinetic data were calculated from the initial velocities using the Michaelis–Menten equation by non-linear regression (KaleidaGraph, Synergy Software). As very low $K_{\text{mA}}^{\text{app}}$ values were indicated by this method, kinetic data were finally determined from progress curves obtained from reactions with initial substrate concentrations of 10 µM. The catalytic rate at each time point, $v(t)$, was obtained by the photometer software from the slope of the absorbance change:

$$v(t) = \frac{[A(t + \Delta t) - A(t)]V}{\Delta t \times \varepsilon} \quad (1)$$

where ε is the extinction coefficient of the ring-cleavage product, V is the volume of the reaction mixture and Δt was 1–2 s.

The substrate concentrations $S(t)$ at each time point were calculated by the amount of ring-cleavage product formed during the further course of the reaction:

$$S(t) = \frac{[A_{\max} - A(t)]V}{\varepsilon} \quad (2)$$

where ε is the extinction coefficient of the ring-cleavage product, V is the volume of the reaction mixture and $A_{\max}$ the absorption after complete substrate transformation. Enzyme concentrations used (approx. 20 nM) ensured that less than 20% of the enzyme was inactivated during the reaction. The datasets obtained were fitted to the Michaelis–Menten equation by non-linear regression (KaleidaGraph, Synergy Software).

The apparent K_{mO_2} for oxygen ($K_{\text{mO}_2}^{\text{app}}$) was determined from progress curves obtained from reactions with initial concentrations of catecholic substrates of 300 µM and 50–60 µM oxygen. The buffer was made anaerobic by alternate vacuum treatment and purging with nitrogen. Eight hundred microlitres of this buffer in 1 ml stoppered

cuvettes was gently mixed with 200 µl air-saturated buffer containing 1.5 mM catecholic substrate. As previously described (Nakajima *et al.*, 2002), the concentration of oxygen in a reaction mixture can be quantified very sensitively based on extradiol cleavage of catecholic substrates. Thus, the total change of absorbance after addition of C23O is a measure of the amount of oxygen present in the reaction. With catechol as substrate, a rapid rise in absorbance of 1.8–2.0 was observed under the above reaction conditions, indicating the formation of 50–55 µM 2-hydroxymuconic semialdehyde. Back-diffusion of oxygen was negligible under the experimental conditions. The catalytic rate at each time point, $v(t)$, was obtained by the photometer software from the slope of the absorbance change, as described above. Oxygen concentrations during the reaction were calculated by the amount of ring-cleavage product formed during the further course of the reaction as described above with:

$$S_{O_2}(t) = \frac{[A_{\max} - A(t)]V}{\epsilon} \quad (3)$$

The enzyme concentrations used (50–80 nM) ensure that less than 20 % of the enzyme was inactivated during the course of the reaction. The datasets obtained were fitted to the Michaelis–Menten equation by non-linear regression (KaleidaGraph, Synergy Software).

Partition ratios (the number of substrate molecules consumed per number of enzyme molecules inactivated) of C23O_{His218} and C23O_{Tyr218} were determined under saturating substrate concentrations (100 µM). The amount of enzyme (approx. 5 nM) was such that the enzyme was completely inactivated before 20 % of the substrate was consumed.

RESULTS

The C23Os of two environmental isolates, *Pseudomonas* sp. strain 1YB2 (capable of using benzene as sole carbon source and containing a C23O enzyme harbouring a tyrosine residue at position 218) and *Pseudomonas* sp. strain 1YdBTEX2 (capable of using benzene, toluene and ethylbenzene as sole carbon and energy source and containing a C23O enzyme harbouring a histidine residue at position 218), were cloned by a new strategy taking advantage of the conserved operon arrangement. Primer sets annealing in conserved regions (Harayama *et al.*, 1987) of the supposed operon neighbouring genes, encoding ferredoxin [e.g. *xylT* in *P. putida* mt-2, *dmpQ* in *Pseudomonas* sp. CF600 (Hugo *et al.*, 2000)] and 2-hydroxymuconic semialdehyde dehydrogenase [e.g. *xylG* in *P. putida* mt-2, *dmpC* in *Pseudomonas* sp. strain CF600 (Harayama *et al.*, 1987; Shingler *et al.*, 1992)] were designed. Single bands of approximately 1.7 kb were observed, indicating that the expected genes were possibly located in operons similar to those of strains mt-2 and CF600. Competent *E. coli* JM109 cells were transformed with these PCR fragments inserted in standard plasmids, and clones expressing C23O were detected and purified. Inserts from plasmids pC23O_{His218} and pC23O_{Tyr218} were sequenced and shown to differ in six nucleotide positions. Five of those differences are located in the third position of a codon, and do not translate into amino acid differences. Thus, the identity of C23O_{His218} along the complete inferred protein sequence to the previously reported C23O gene of *P. putida* AN10

was confirmed, as well as the presence of only a single amino acid difference in C23O_{Tyr218}. By a similar strategy using primers annealing in neighbouring genes, complete and functional genes could also be isolated directly from environmental DNA (data not shown). This straightforward strategy avoids the introduction of artificial mutations as a consequence of the generation of PCR products from primers that anneal to mismatches in coding regions.

Cell extracts of *E. coli* JM109 (pC23O_{His218}) expressing C23O_{His218} and *E. coli* JM109 (pC23O_{Tyr218}) expressing C23O_{Tyr218} were analysed by SDS-PAGE, and a prominent band of 35 ± 2 kDa, which was absent in cell extracts of *E. coli* JM109 (pGEM-T), was observed (Fig. 1). The identity of the bands with C23O_{His218} and C23O_{Tyr218} was confirmed by N-terminal sequencing and MALDI-TOF analysis. The relative amounts of these C23O protein bands were 29 ± 2 % of the total protein content in each case, showing that expression levels of the two C23O variants did not differ significantly (Fig. 1). As C23Os are known to be subject to inactivation during protein purification due to oxidation and/or loss of active site ferrous iron (Eltis *et al.*, 1993; McKay *et al.*, 2003), kinetic data were measured directly in these cell extracts. Spontaneous inactivation in these extracts was negligible: <10 % in 8 h. Moreover, spontaneous oxygen consumption was negligible, such that no interference with kinetic measurements can be expected.

As C23O proteins comprise 29 ± 2 % of the total protein content in cell extracts (Fig. 1), it can be calculated that maximum turnover rates with catechol of 63 700 and 18 800 U protein g⁻¹ in C23O_{His218}- and C23O_{Tyr218}-containing cell extracts correspond to activities of 219 500 and 64 700 U C23O g⁻¹ (Table 1) and thus, based on sub-unit molecular masses of 35 050, to k_{cat} values of 128 s⁻¹ and 38 s⁻¹, respectively. This indicates a significantly

Fig. 1. Separation by SDS-PAGE of proteins from cell extracts of *E. coli* expressing recombinant C23Os. Gels were stained with ruthenium II tris (bathophenanthroline disulfonate) as previously described (Rabilloud *et al.*, 2001). Lane M, low-range molecular mass marker (Bio-Rad, 8.8 µg protein); lanes 1–4, 15.0, 3.0, 1.5 and 0.3 µg, respectively, of total protein from cell extract of *E. coli* pC23O_{Tyr218}; lanes 5–8, 15.0, 3.0, 1.5 and 0.3 µg, respectively, of total protein from cell extract of *E. coli* pC23O_{His218}. The arrow indicates proteins identified by N-terminal sequencing and MALDI-TOF analyses as C23O.

Table 1. Apparent kinetic parameters of the catechol 2,3-dioxygenases C23O_{Tyr218} and C23O_{His218}

Experiments were performed with potassium phosphate buffer, pH 7.5, at 25 °C. Each value represents the mean of at least three determinations. Kinetic parameters for catechols were measured in air-saturated buffer. *J* represents the apparent rate constant of inactivation of C23O by catechols. Kinetic parameters for oxygen were measured with saturating but non-inhibiting concentrations of catechols. $K_{mO_2}^{app}$ represents the apparent K_m for oxygen in the presence of the respective catechol cosubstrate. ND, Not determined.

Enzyme	Substrate	Activity (U g ⁻¹)*	k_{cat} (s ⁻¹)†	K_{mA}^{app} (μM)	k_{cat}/K_{mA}^{app} (μM ⁻¹ s ⁻¹)	Partition ratio‡	<i>J</i> (10 ⁻³ s ⁻¹)§	$K_{mO_2}^{app}$ (μM)
C23O _{Tyr218}	Catechol	64 700 ± 4 100	38 ± 2	1.1 ± 0.2	35	ND	ND	8 ± 2
	3-Methylcatechol	29 200 ± 2 000	17 ± 1	1.5 ± 0.2	11	4000 ± 500	4.2	16 ± 4
	4-Methylcatechol	90 900 ± 7 300	53 ± 4	1.9 ± 0.2	28	6200 ± 600	8.5	11 ± 3
	4-Chlorocatechol	29 400 ± 2 100	17 ± 1	0.9 ± 0.2	19	3300 ± 400	5.2	17 ± 4
C23O _{His218}	Catechol	219 500 ± 14 100	128 ± 8	2.5 ± 0.2	51	ND	ND	10 ± 2
	3-Methylcatechol	42 800 ± 2 900	25 ± 2	3.2 ± 0.3	8	4300 ± 500	5.8	40 ± 12
	4-Methylcatechol	109 900 ± 7 600	64 ± 4	5.2 ± 0.4	12	6000 ± 600	10.7	14 ± 3
	4-Chlorocatechol	77 200 ± 5 800	45 ± 3	1.6 ± 0.2	28	6800 ± 800	6.6	42 ± 10

*Activities are given per gram of C23O, assuming C23O to comprise 29 ± 2% of the total protein in the cell extract.

†Values were calculated from the activity per gram of C23O, assuming subunit molecular masses of 35 050.

‡The partition ratio is defined as the number of substrate molecules consumed divided by the number of enzyme molecules inactivated.

§Values were calculated by dividing k_{cat} by the partition ratio (Vaillancourt *et al.*, 2002).

increased turnover rate of C23O_{His218}. Such a difference is of the same order of magnitude as that previously described for the site-directed mutants of an extradiol dioxygenase involved in tetralin degradation, for which turnover numbers could be increased by a factor of three compared to the wild-type (Andujar & Santero, 2003).

Less-pronounced differences between the enzyme variants were observed for the turnover of substituted catechols, even though C23O_{His218} usually exhibited higher turnover numbers. Thus, a dominant effect of the variation is the increased turnover rate for catechol. In comparison to C23O_{His218} and C23O_{Tyr218}, C23O_{mt-2} (C23O Xyle of *P. putida* mt-2) showed a k_{cat} value of 420 s⁻¹ (Wasserfallen *et al.*, 1991) for catechol. A second difference lies in the lower apparent K_m values for all tested catecholic substrates of C23O_{Tyr218}.

C23O enzymes are characterized by their sometimes rapid inactivation through oxidation of active site iron during catalytic turnover. As an example, the partition ratio of C23O_{mt-2} with 4-ethylcatechol was reported to be 6500, whereas that of 3-methylcatechol was 210 000 (Cerdan *et al.*, 1994). Whereas there was no significant difference in partition ratio between the variants (Table 1), the partition ratios observed with all three substituted catechols analysed were dramatically lower than those observed for C23O_{mt-2}.

The affinity for oxygen is another parameter critical to the activity of C23Os. Both enzymes exhibited $K_{mO_2}^{app}$ values of 8–10 μM in the presence of saturating concentrations of catechol (Table 1). These values are slightly higher than those reported for C23O_{mt-2} (3.9 μM) (Kukor & Olsen,

1996). $K_{mO_2}^{app}$ values for oxygen in the presence of saturating concentrations of 3-methyl-, 4-methyl- or 4-chlorocatechol varied only slightly (by a factor of 2) in the case of C23O_{Tyr218}. However, in comparison, $K_{mO_2}^{app}$ values for oxygen in the presence of saturating concentrations of 4-chlorocatechol and 3-methylcatechol were significantly increased in the case of C23O_{His218}.

Evidently, the nature of the amino acid at position 218 has a prominent effect on the enzyme kinetics. In previously described C23Os, this position is usually occupied by histidine or phenylalanine and, exceptionally, leucine (Fig. 2). A tyrosine in this position was only observed in a thermostable chimeric gene, which contained a central C23O gene fragment from an environmental sample (Okuta *et al.*, 1998). This position was, however, never considered relevant for enzyme functioning. To understand possible influences of histidine/tyrosine variants on enzyme function, three-dimensional models of the two protein types were constructed, based on the available crystal structure of the homologous and highly similar (identities 290/307, 94%) Xyle protein (C23O_{mt-2}) (Kita *et al.*, 1999) (Fig. 3). Amino acid 218 is not involved in the formation of the substrate-binding pocket, nor does it interfere with the hydrophobic channel through which catechols and dioxygen are thought to enter the active site (Kita *et al.*, 1999). This amino acid is localized on the side of the molecule, which is buried in the tetramer interface, and appears to be part of a second smaller channel leading to the active centre. The functional role of this channel (Fig. 3) is unclear, and His/Tyr218, with a distance of more than 9 Å to the active iron and pointing away from it, is unlikely to interfere much with water or oxygen molecules filling the channel.

		200	210	220	230	
						
AAM54735	196		C	F		235
P17262	196		C	F		235
JC4137	197		T	F	R	236
BAA11757	196		C	F		235
BAC75709	195		C	F		234
S47421	196		C	F		235
CAD67847	183		C	F		222
S42100	196		C	F	R	235
Q04285	196		A	F		235
CAE46794	145			F		184
JC4885	196	P		H		235
P27887	196			H		235
AAD02148	196			H		235
C230 _{His218}	196	TKAHDVAFIHHPEKGRLLHVSF	LETWEDVLR	AAADLISMT		235
C230 _{Tyr218}	196	TKAHDVAFIHHPEKGRLLHVSF	LETWEDVLR	AAADLISMT		235
BAA31264	196		C	F		235
NP_542866	196	TKAHDVAFIHHPEKGRLLHVSF	LETWEDVLR	AAADLISMT		235
AAF36683	196			L		235
CAD43168	196			H		235
CAD67851	183			H		222
AAC78337	196			H	L	235
BAA11752	196			H		235
JC5654	196	P		H		235
AAA20982	196			H		235
NP_863103	196		A	L	D	235
CAD67834	183		A	L	D	222
BAA31718	196		T	L		235
BAA31719	196		T	F	L	235
BAA31715	196		A	F		235
P08127	196		A	F	D	235
BAA31717	196		A	P	Y	235
BAA31716	196			F	D	235
BAB92969	196		A	F	I	235
ZP_00092918	196 M	E		F		235
BAA31720	196 M	D	P	F		235
BAA23555	196			F		235
Consensus		TKAHDVAFIHHPEKGRLLHVSF	LETWEDVLR	AAADLISMT		
		QC	KF	A	F	

Fig. 2. Alignment of 36 C23O subfamily I.2.A proteins. A selected block spanning 39 amino acids in the neighbourhood of residue 218 is shown. Complete sequences used for the alignment (>60% identity) were obtained from GenBank/EMBL/DBJ databases (respective accession numbers are given). Sequences are arranged (top to bottom) according to global alignment output (total similarity), using default values of the CLUSTAL X (1.8) program. Amino acid sequences of *P. putida* mt-2 XylE (NP_542866), *Pseudomonas* sp. strain 1YB2 C23O (C23O_{Tyr218}) and *Pseudomonas* sp. strain 1YdBTEX2 C23O (C23O_{His218}) are shown in white type on a grey background. Amino acids observed at the respective position in fewer than 20% of the sequences are shown in black type, whereas conserved amino acids or amino acids present at the respective position in at least 20% of the sequences are indicated at the bottom of the figure (consensus). Amino acids present at position 218 are shown for all the sequences. In this case, amino acids present in at least 35% of the sequences are shown in white type on a black background.

DISCUSSION

Until now, only a few studies had analysed the effect of single amino acid mutations on the functioning of C23Os. Wasserfallen *et al.* (1991) screened for mutant C23O_{mt-2}, which allowed the *Pseudomonas* host to grow on 3-methylbenzoate in the presence of 3-chlorocatechol, a substrate mixture with which the original host cannot deal due to rapid inactivation of C23O_{mt-2} during 3-chlorocatechol turnover. A point mutation in *xylE*, which led to the substitution of Val291 for Ile291, enabled the host

Fig. 3. Location of the His/Tyr218 (black/red carbon atoms) relative to the active centre of C23O. In the active centre, the iron atom (brown sphere) with the bound His214 and Glu265 is shown. The substrate-binding pocket is indicated by the acetone molecule observed in the C23O structure 1MPY (Kita *et al.*, 1999) inside a semitransparent surface connected to the outside of the molecule on the top right. The His/Tyr218 points to the tetramer interface (magenta and yellow tubes, bottom and right) at the entrance of a small channel leading from the interface to the active centre. The structural model was generated from the C23O crystal structure (pdb-ID: 1MPY) by manual replacement of the few different amino acids with the best-fitting side-chain rotamer using 'O' (Jones *et al.*, 1991) and a CLUSTAL X 1.8 alignment of the two C23O protein sequences analysed in this study. The image was prepared with Molscript (Kraulis, 1991) and GRASP (Nicholls *et al.*, 1991), and rendered with POV-Ray (www.povray.org).

to grow on this mixture. The mutant enzyme was characterized by an increased apparent K_m for 3-chlorocatechol, a decreased apparent K_m for 3-methylcatechol, and higher partition ratios for substituted catechols, specifically 4-ethylcatechol. A set of C23O_{mt-2} mutants has been isolated by a similar strategy (selection for growth on 4-ethylbenzoate) for their capability to transform 4-ethylcatechol (Cerdan *et al.*, 1994; Ramos *et al.*, 1987) and, like the Val291Ile mutant, Leu226Ser and Thr253Ile substitutions increased the catalytic reaction with 4-ethylcatechol. As the mutants exhibited reduced binding of the ferrous ion cofactor, it was assumed that the substitutions significantly modified the substrate-binding pocket (Cerdan *et al.*, 1994). A further mutant, Ala177Val, was isolated based on its lowered partition ratio with 3-methylcatechol as substrate, and a further Thr196Ile mutant reversed this effect. The effect of all the above single amino acid substitutions on enzyme kinetic parameters was

less than one order of magnitude. Astonishingly, none of the above mutations, all isolated based on a clear change of the phenotype of the host strain, was localized in the substrate-binding pocket. Whereas the exchange of Thr253 to Ile can be assumed to result in a minor effect on the active site by abolishing a hydrogen bond participating in the stabilization of the Glu265 side chain, which is bound to the active iron, and whereas Val291 is situated in the inner channel wall of the active site pocket (Kita *et al.*, 1999), Leu 226 and Ala 177 are situated near to the subunit interface, such that no direct influence of these amino acids on the active site can be envisaged. Similarly, a direct effect on the substrate-binding pocket of a His to Tyr exchange at position 218 is not evident. However, various examples of mutant enzymes altered in their catalytic properties, predictable with difficulty by rational design, have been reported in studies of the directed evolution of proteins. Only a careful analysis of structural changes in a set of *p*-nitrobenzyl esterase mutants identified networks of mutations that collectively remodel the active site (Spiller *et al.*, 1999), and it is a matter of fact that various proteins are altered in their kinetic properties by mutations in non-active-site residues (Kraut *et al.*, 2003; Que & Ho, 1996; Shimotohno *et al.*, 2001; Zielinski *et al.*, 2003). Some mutations may not alter the mean structure of the protein as determined by crystallography, but may exert an effect on the enzymic activity of the protein by changing its dynamics (Yang *et al.*, 2003) or create small structural changes affecting protein kinetics by long-range interactions which are difficult to predict.

Interestingly, several characteristics distinguish the naturally occurring Tyr218 and His218 variants. The Tyr218 variant, detected both in highly and slightly contaminated soil, and present in benzene degraders, exhibits low turnover number and high affinity, whereas the His218 variant, detected in highly contaminated sites only, and present in toluene/benzene degraders, exhibits a high turnover number and low affinity. As such, the variants seem to be selected for by the environmental conditions. Whether the different growth phenotypes of the strains harbouring the Tyr218 and His218 variants are actually a consequence of the different kinetic characteristics of the enzymes or result from other characteristics of the host will need to be analysed by site-directed mutagenesis in the same strain.

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