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Protocatechuate 4,5-dioxygenase from *Comamonas testosteroni* T-2: biochemical and molecular properties of a new subgroup within class III of extradiol dioxygenases

Abstract *Comamonas testosteroni* T-2 degraded at least eight aromatic compounds via protocatechuate (PCA), whose extradiol ring cleavage to 2-hydroxy-4-carboxymuconate semialdehyde (HCMS) was catalysed by PCA 4,5-dioxygenase (PmdAB). This inducible, heteromultimeric enzyme was purified. It contained two subunits, α (PmdA) and β (PmdB), and the molecular masses of the denatured proteins were 18 kDa and 31 kDa, respectively. PCA was converted stoichiometrically to HCMS with an apparent K_m of 55 μM and at a maximum velocity of 1.5 μkat. Structure–activity-relationship analysis by testing 16 related compounds as substrate for purified PmdAB revealed an absolute requirement for the vicinal diol and for the carboxylate group of PCA. Besides PCA, only 5′-hydroxy-PCA (gallate) induced oxygen uptake. The N-terminal amino acid sequence of each subunit was identical to the corresponding sequences in *C. testosteroni* BR6020, which facilitated sequencing of the *pmdAB* genes in strain T-2. Small differences in the amino acid sequence had significant effects on enzyme stability. Several homologues of *pmdAB* were found in sequence databases. Residues involved in substrate binding are highly conserved among the homologues. Their sequences grouped within the class III extradiol dioxygenases. Based on our biochemical and genetic analyses, we propose a new branch of the heteromultimeric enzymes within that class.

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Keywords Extradiol · Oxygenase · Degradation · Toluenesulfonate · Structure–activity relationship

Introduction

Oxygenases play key roles in aerobic degradation of natural or xenobiotic aromatic compounds by (1) activation (ring hydroxylation) and (2) cleavage of the inherently inert aromatic structure. A broad variety of peripheral pathways channel aromatic substrates to a limited number of central diol intermediates which are further metabolized by enzymes of central pathways, allowing the generation of amphibolic intermediates. For vicinal diols like catechol and protocatechuate (PCA), ring-cleavage dioxygenases catalyse the conversion of aromatic substrates into aliphatic products by intradiol fission of the C–C bond (*ortho* cleavage) or extradiol fission (*meta* cleavage).

It is generally accepted that extradiol oxygenases are evolutionarily unrelated to intradiol cleavage enzymes (Harayama et al. 1992). Extradiol dioxygenases of classes I and II were first distinguished by biochemical data (Harayama and Rekik 1989). Molecular data showed that class II enzymes, with their two-domain structure, evolved by gene duplication from ancestral, one-domain, class I extradiol enzymes (Eltis and Bolin 1996). In contrast, class III enzymes do not share a common ancestor with enzymes of the other two classes. Their structural fold bears no resemblance to the fold shared by class I and class II enzymes (Sugimoto et al. 1999).

Dagley et al. (1968) cited the widespread importance of the extradiol cleavage of PCA and inferred biodiversity in the extradiol cleavage enzymes (Dagley et al. 1960). PCA is the point of convergence of pathways for degradation of many aromatic compounds, for example *p*-toluenesulfonate in *Comamonas testosteroni* T-2 (Cook et al. 1999), of *m*-chlorobenzoate in *C. testosteroni* BR60 (Nakatsu and Wyndham 1995), and of lignin

in *Sphingomonas paucimobilis* SYK-6 (Noda et al. 1990; Hara et al. 2003). In these bacteria, PCA is degraded by PCA 4,5-dioxygenase (PmdAB), which is encoded by *ligAB* in *S. paucimobilis* SYK-6 and *pmdAB* in *C. testosteroni* BR6020 (Providenti et al. 2001). The crystal structure of LigAB from *S. paucimobilis* SYK-6 (Sugimoto et al. 1999) was preliminary evidence that this group of extradiol cleavage enzymes could be attributed to class III; however, there was a need to broaden the available data set to support this notion.

Transport, regulation and the soluble enzymes of the peripheral pathway in the degradation of *p*-toluenesulfonate and *p*-toluenecarboxylate to PCA by *C. testosteroni* T-2 have been explored, and all oxygenases involved were characterized except for PmdAB (Cook et al. 1999; Mampel 2000; Mampel et al. 2004; Tralau et al. 2003a, 2003b). We now present an analysis of this class III oxygenase from *C. testosteroni* T-2 which catalyses the key reaction linking peripheral and central degradative pathways. This work further illustrates biodiversity among class III oxygenases in terms of phylogenetic relationships and biochemical properties, although key features of these enzymes appear highly conserved.

Materials and methods

Bacteria, growth conditions, harvesting of cells and preparation of heat-treated, cell-free extract

The following strains of *C. testosteroni* were used: the type strain ATCC 11996, BR6020 (Providenti et al. 2001), PSB-4 (DSM 11414) (Thurnheer et al. 1986) and T-2 (DSM 6577) (Thurnheer et al. 1986; Busse et al. 1992). *C. testosteroni* was grown at 30°C in mineral salts medium (Thurnheer et al. 1986; Junker et al. 1996) containing a sole carbon source at 6 mM initial concentration on a 1.5-l scale, in 5-l shake flasks at 225 rpm. Strain T-2 for enzyme purification was grown at 30°C in a 12.5-l fermenter with a 9-l working volume (Biostat V, Braun, Melsungen) in 12 mM PCA-salts medium (Thurnheer et al. 1986; Junker et al. 1996). Cells were harvested at 580 mg protein/l (see 'Results') in a Pellikon cassette filtration system (Millipore), washed twice in ice-cold 50 mM potassium phosphate buffer (pH 7.5) and stored frozen (−20°C).

Cells (25 g wet weight) were resuspended in 25 ml chilled, freshly prepared 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and DNase I (2 μg/ml), and disrupted by three passages through a chilled French pressure cell at 135 MPa. Phenylmethanesulfonylchloride (to 1 mM), L-cysteine (to 1 mM) and $Fe(NH_4)_2(SO_4)_2$ (to 50 μM) were added, and whole cells and debris were removed by centrifugation (36,000 *g*, 30 min, 4°C). The stirred supernatant fluid was then brought to 45°C for 2 min and immediately chilled in a cryostat. Precipitated proteins and membrane fractions were removed by ultracentrifugation (500,000 *g*, 40 min, 4°C). The supernatant fluid (50–70 mg protein/ml) was filtered (0.2-μm pore diameter) and could be stored frozen at −70°C for at least 4 weeks, without significant loss of activity.

Enzyme assays

The activity of PmdAB was assayed routinely at 30°C as substrate-dependent oxygen uptake in a Clark-type oxygen electrode (Locher et al. 1991). Reaction mixtures (0.5 ml) contained 40 mM Tris-SO_4 (pH 7.5), 0.5–1.5 mg protein and 2.5 μmol PCA, with which the reaction was started. Enzyme kinetics were determined by substrate induced oxygen uptake assays. The PCA concentrations applied were 3–600 μM; K_m and V_{max} values were derived from nonlinear regression analysis of Michaelis–Menten plots, using a two-parameter fitting (SigmaPlot). The resulting equation was $f = a^*[1-\exp(-b^*x)]$, $a = 88.6$, $b = 0.0125$; $R^2 = 0.97$. PmdAB in fractions from column chromatography was detected as the increase in absorbance at 410 nm at 25°C, due to the formation of product (Ribbons and Evans 1960). Positive fractions could be detected visually and quantified after 5 min. The 1-ml reaction mixture contained 4 mM PCA in 20 mM Tris/SO_4 (pH 7.0), and the reaction was started with 50–150 μl column eluent.

Screening for PmdAB in differently grown cells

Organisms were grown with different carbon sources, harvested, disrupted, and the supernatant fluid used without further treatment. About 80 mg protein was loaded on to an anion-exchange column (UnoQ, 6-ml bed volume, Bio-Rad) equilibrated with 20 mM Tris/SO_4 (pH 7.5) containing 1 mM DTT (buffer A) at 2 ml/min, and fractions of 4.5 ml were collected. The column was washed with buffer A for 30 min, when a linear gradient to 20% buffer B [20 mM Tris/SO_4 (pH 7.5) 1 M Na_2SO_4, 1 mM DTT] was applied for 63 min and then ramped to 100% B in 10 min. Each fraction was assayed visually for the presence of PmdAB as an increase in absorbance at 410 nm. Activity was confirmed and quantified as PCA-dependent oxygen uptake. Proteins in the fractions with the highest specific activity were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of PmdAB

A three-step chromatographic procedure was used, which was carried out in one day. Fractions containing significant activity were combined, and the protein in this solution was desalted and concentrated by membrane filtration (30-kDa exclusion limit, Diaflo, Amicon) in a stirring cell (model 8050, Amicon). The desalting buffer was 10 mM Tris/SO_4 buffer containing 1 mM

DTT, 2 mM L-cysteine, 50 μM $Fe(NH_4)_2(SO_4)_2$, 5% (v/v) glycerol and 2.5 mM ascorbate and adjusted to pH 7.5. All mobile phases were freshly prepared and degassed in an ultrasonic bath before oxidation-sensitive ingredients were added. Mobile phases included 1 mM DTT, 2 mM L-cysteine, 50 μM $Fe(NH_4)_2(SO_4)_2$ and 5% (v/v) glycerol. They were degassed and subsequently protected against atmospheric oxygen by a blanket of argon. Fast protein liquid chromatography was done at room temperature with a Pharmacia apparatus. Samples for flash freezing in liquid nitrogen contained glycerol (20% v/v); they were stored at −70°C. The three-step chromatographic procedure proceeded as follows:

1. Heat-treated extract (about 500 mg protein) was loaded on the DEAE-column (300×26 mm, DEAE-Sepharose CL-6B, Pharmacia), which was run with supplemented (see above) Tris-buffered eluents (pH 7.5), as described elsewhere (Junker et al. 1994b). The fraction size was 5 ml, and the enzyme eluted between 275 ml and 315 ml.
2. Gel filtration chromatography was done with a Superformance cartridge (600×10 mm, Merck) filled with Superdex 200 prep grade material (Pharmacia). The flow rate was 0.8 ml/min of supplemented 50 mM Tris/SO_4 buffer (pH 7.5) containing 150 mM NaCl. Activity-containing fractions (fraction size 0.8 ml) eluted between 22 ml and 25 ml.
3. A commercial anion exchange column (Resource Q, 1-ml bed volume; Pharmacia) was equilibrated with supplemented 20 mM Tris/SO_4 (pH 7.5) at a flow rate of 1 ml/min. The fraction size was 0.5 ml. The sample was loaded and the column rinsed for 5 min before a linear gradient to 10% high-ionic-strength buffer [supplemented 20 mM Tris/SO_4 (pH 7.5) 1 M Na_2SO_4] was applied over the next 2 min. The gradient was then raised to 20% high-ionic-strength buffer in 5 min and finally increased to 100% high-ionic-strength buffer in 5 min. The enzyme eluted between 11 min and 13.5 min.

Analytical methods

Growth was estimated as turbidity (optical density at 580 nm) and quantified as protein in a Lowry-type reaction after solublizing the bacteria (Kennedy and Fewson 1968). PCA and 2-hydroxy-4-carboxymuconate semialdehyde (HCMS) were separated by reversed-phase high-performance liquid chromatography (HPLC) (Locher et al. 1989; Laue et al. 1996). Protein was routinely determined by the method of Bradford (Bradford 1976), with bovine serum albumin as standard. The molecular mass of native PmdAB was assayed by gel-filtration chromatography on a Superose 12 column (Pharmacia) with the mobile phase described in step 2 of the gel filtration chromatography procedure (see above) at a flow rate of 0.4 ml/min; standard proteins are described elsewhere (Locher et al. 1991). SDS-PAGE was done with 12% separative gels (Schägger and von Jagow 1987), and proteins were stained with colloidal Coomassie Brilliant Blue G-250 (Neuhoff et al. 1988); a 10-kDa Protein Ladder (Gibco) was used for calibration. Proteins were subject to blotting and N-terminal sequencing, as described elsewhere (Laue and Cook 2000).

Polymerase chain reaction (PCR), reverse transcriptase-PCR, DNA sequencing and sequence analysis

PCR quantification of DNA and cycle sequencing was done as described elsewhere (Laue et al. 2001; Tralau et al. 2001). Reverse transcriptase (RT)-PCR of *p*-toluenesulfonate- and PCA-grown cells was essentially done as described by Tralau et al. (2003a). Oligonucleotides to sequence *pmdAB* in *C. testosteroni* T-2 by primer walking were derived from the nucleotide sequence of the *pmd* locus in *C. testosteroni* BR6020 (AF305325; Providenti et al. 2001). The following primers were used to detect by PCR *pmdA, pmdB* and *pmdAB* in different organisms: pmdA30 (5′-GCCCGGCACCATCATTTT-3′), pmdA428 (5′-AGTTGCCTTCCTGGTTCTGGT-3′), pmdB5830 (5′-TCTCGCGCCAGTGGATGAAGGACAACAA-3′) and pmdB6450 (5′-GCACCACGCGCAATCAGCCACATCAC-3′). Oligonucleotide pmdB6450 was the primer for reverse transcription of mRNA for *pmdB*, and primer pair pmdB5830/pmdB6450 was used for subsequent PCR amplification of cDNA. Primer pairs, annealing temperature and PCR-product length were as follows:

- Amplification of *pmdA*: pmdA30 and pmdA428, 54°C, 421 bp.
- Amplification of *pmdB*: pmdB5830 and pmdB6450, 65°C, 648 bp.
- Amplification of *pmdAB*: pmdA30 and pmdB6450, 54°C, 1,187 bp.

Sequence data were analysed using standard software (Edit View from PerkinElmer and DNAStar package from Lasergene). Database searches were done with BLAST (Altschul et al. 1997). Multiple sequence alignments were generated by the Clustal X program (Jeanmougin et al. 1998). Relative phylogenetic distances were estimated and visualized using the Megalign program (Clustal method) of the DNAStar package and the Markov program of Saccone et al. (1990, 1993); the latter was also used to estimate the phylogenetic branch points during common evolution of extradiol oxygenases.

Nucleotide sequence accession number

The nucleotide sequence reported in this study has been deposited to the GenBank DNA database under accession no. AF459635.

Results

Basal expression and induction of a single PmdAB during growth of *C. testosteroni* strains with aromatic compounds

Extracts of succinate-grown cells of *C. testosteroni* T-2 contained a basal level of PmdAB (Table 1), some 5–10% of the activity found in induced cells, which contained the enzyme at 2.2–5.5 mkat/kg protein (Table 1). The chromatographic properties of the enzyme from each extract on an ion exchange column were essentially identical (Table 1), so we believe that the same gene products were assayed in each case, as predicted from genetic evidence for a single set of *pmdAB* genes from a different strain of *C. testosteroni* (Providenti et al. 2001). The active fraction from each chromatogram was subject to SDS-PAGE (not shown), and each sample contained bands corresponding to the α and β subunits of the enzyme (see below). The enzyme from two other strains of *C. testosteroni* had the same chromatographic properties as PmdAB (Table 1), the predicted subunits (SDS-PAGE; not shown), and tested positive by PCR for the presence of *pmdA* and *pmdB*.

PmdAB from strain T-2 converted PCA to a yellow compound, which Locher et al. (1989) tentatively identified as HCMS (Fig. 1); the compound was colourless in acid and had the anticipated UV spectrum (cf. Dagley et al. 1960). We confirmed the identification by allowing HCMS to react with ammonium ion and form 2,4-lutidinate, which was confirmed by co-chromatography (HPLC) and identical UV spectra (λ_{max}, 207 and 283 nm; shoulder, 227 nm; λ_{min}, 252 nm) (cf. Dagley et al. 1960).

Table 1 Activity of protocatechuate 4,5-dioxygenase (PmdAB) in crude extracts of *Comamonas testosteroni* strains grown with different sources of carbon and energy, and elution of the enzyme from an anion exchange column

Strain	Carbon source	Specific activity (mkat/kg protein)	Active fraction
T-2	Succinate	0.2	33
T-2	Protocatechuate	2.5	33
T-2	*p*-Toluenesulfonate	2.7	33
T-2	*p*-Toluenecaboxylate	2.5	33
T-2	Terephthalate	5.5	35
T-2	*p*-Sulfobenzoate	4.3	36
T-2	*p*-Anisate	2.2	36
T-2	Vanillate	3.8	35
T-2	Phthalate	3.6	34
PSB-4	Protocatechuate	2.2	34
ATCC 11996	Protocatechuate	4.3	34

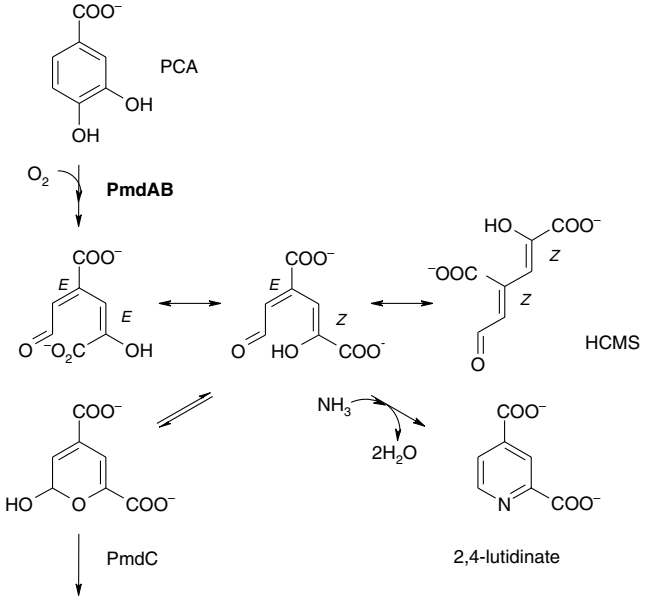

Fig. 1 The reaction of protocatechuate 4,5-dioxygenase (*PmdAB*) and the nonenzymic reactions leading to the next step in the pathway (*PmdC*) or nonenzymic formation of lutidinate

Specific activity of PmdAB in *C. testosteroni* T-2 as a function of the growth phase

The growth yield of strain T-2 was a linear function of the substrate concentrations up to at least 12 mM PCA, the concentration which was used routinely. The maximum specific growth rate was 0.31/h (Fig. 2), and growth was concomitant with substrate utilization; correspondingly, no degradative intermediates were detected in the growth medium. The molar growth yield was 5.2 g protein/mol carbon, a normal value corresponding to complete dissimilation of the substrate (Cook 1987).

The specific activity of PmdAB varied widely during growth (Fig. 2). The enzyme was not detectable at the start of the experiment, but activity rose steadily to

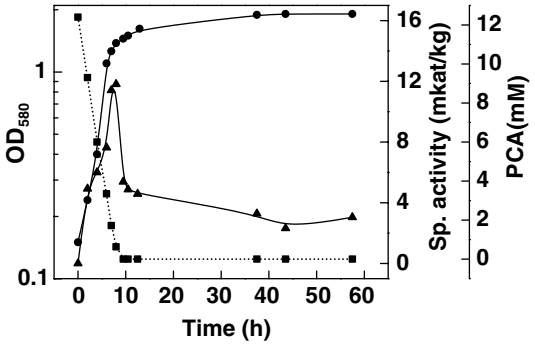

Fig. 2 Growth, substrate utilization and specific activity of PmdAB during growth of *C. testosteroni* T-2 in 12 mM protocatechuate (*PCA*)-salts medium. The inoculum was taken from an exponentially growing culture in PCA-salts medium. Growth (*filled circles*) was assayed as turbidity. The specific activity (mkat/kg protein) of the enzyme in whole cells (*filled triangles*) was the PCA-dependent oxygen uptake. Substrate concentration (*filled squares*) was determined by high-performance liquid chromatography

12 mkat/kg protein, when the growth rate was decreasing (about 12 h, Fig. 2), only to fall to about 5 mkat/kg protein in the stationary phase, where it was effectively stable. Stability of PmdAB activity was independent of ongoing transcription as demonstrated by RT-PCR analysis, which showed the absence of mRNA encoding *pmdB* in stationary phase. We chose to harvest the cells at a turbidity of 1.1 (580 mg protein/ml), when the specific activity of the enzyme was still rising.

Purification of PmdAB from *C. testosteroni* T-2

We attempted to purify PmdAB, using essentially the protocol for *C. testosteroni* Pt-L5 (Arciero et al. 1983), but after the heat treatment, which yielded a purification (Table 2), hydrophobic interaction chromatography (Phenyl Superose HR) and anion exchange chromatography (UnoQ) caused complete loss of activity. We then supplemented the mobile phases with glycerol, three reducing agents and Fe^{2+}, and protected solutions from atmospheric oxygen under a blanket of argon. We chose DEAE-Sepharose and Superdex 200 material for the first two chromatographic steps and involved a strongly interacting column (ResourceQ) for the final step only (Spence et al. 1996). Freezing and thawing were avoided to prevent precipitation of protein; further steps led to loss of activity. We were thus able to purify the labile enzyme to over 80% purity, judged by densitometric analysis of SDS-PAGE electropherograms (Fig. 3). This was a 184-fold purification with a final yield of 5% (Table 2). PmdAB thus represents a very small proportion of soluble cell protein (0.05%).

Physical properties of PmdAB from *C. testosteroni* T-2

The enzyme was presumed to contain two subunits, α (18 kDa, PmdA) and β (31 kDa, PmdB), and the preparation contained a significant impurity (29 kDa, Fig. 3, lane 5). The molecular mass of the native enzyme was estimated by gel filtration to be 180 kDa. The enzyme was thus a heteromultimer. Due to sequence and structural similarity to LigAB (see below), we suspect that in contrast to LigAB, PmdAB has an $(\alpha\beta)_4$ structure.

The N-terminal sequence of PmdA was Ala-Leu-Glu-Lys-Pro-Tyr-Leu-Asp-Val-Pro-Gly-Thr-Ile. The N-terminal sequence of PmdB was Ala-Arg-Ile-Thr-Ala-Ser-Val-Phe-Thr-Ser-His-Val-Pro. The amino acid sequence of PmdAB in strain T-2 deduced from the gene sequence (see below) includes those obtained by protein microsequencing and show that in each polypeptide, the N-terminal Met was absent from the purified protein. These sequences are identical with those from $PmdAB_{BR6020}$ (Providenti et al. 2001).

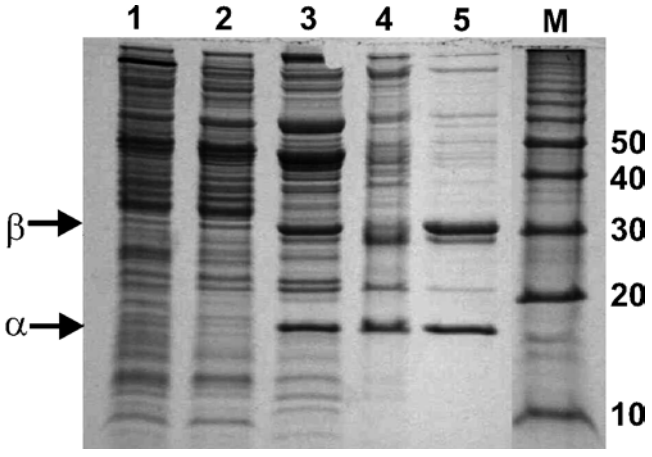

Fig. 3 Purification of PmdAB from *C. testosteroni* T-2 monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Protein (30 µg, *lanes 1–3*; 15 µg, *lanes 4–6*) was separated and subject to staining with Coomassie Brilliant Blue. *Lane 1* Crude extract, *lane 2*, supernatant after heat treatment, *lane 3* fraction from anion exchange chromatography, *lane 4* fraction from gel filtration, *lane 5* fraction from the second anion exchanger, *M* molecular mass standards (in kilodaltons)

Catalytic properties of PmdAB from *C. testosteroni* T-2

The stoichiometry of oxygen required to convert PCA to HCMS was 1:1 when the initial concentration of PCA was <120 µM. Above this concentration, the reaction did not go to completion, as has been observed previously (Dagley et al. 1968). We derived values of 55.5 (± 2.3) µM PCA and 1.47 (± 0.3) µkat for apparent K_m and V_{max}, respectively; this is close to the value (46 µM) for K_m reported for *C. testosteroni* NCIMB 8893 (Dagley et al. 1968).

We tested the substrate range of PmdAB with structural analogues of PCA: benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, catechol, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxyphenylacetate, 2,3-dihydroxybenzoate,

Table 2 Purification of PmdAB from *C. testosteroni* T-2

Fraction	Total protein (mg)	Total activity (nkat)	Specific activity (mkat/kg protein)	Yield (%)	Purification
Crude extract	1,138	11.5	9	100	1
Heat treatment	435	10.8	25	102	2.7
DEAE-Sepharose	21.2	2.1	99	20	10.7
Superdex 200	5.8	5.2	894	49	96.4
Resource Q	0.3	0.5	1,709	5	184

3,4,5-trihydroxybenzoate (gallate), 2,4-dihydroxybenzoate, 3,5-dihydroxybenzoate, 2,6-dihydroxybenzoate, gentisate, 3,4-dichlorobenzoate, vanillate and 3,4-diaminobenzoate. Only gallate was a substrate. Thus, the vicinal diol in the 3,4-position and the carboxylate group are essential for activity. In contrast to findings for PmdABs from other sources (Zabinski et al. 1972; Arciero and Lipscomb 1986), none of these nonsubstrates inhibited turnover of PCA, which indicates a high selectivity of the active site in PmdAB in strain T-2. We presume that $PmdAB_{T\text{-}2}$ will also oxidatively cleave (and dechlorinate) 5-chloroprotocatechuate, as observed elsewhere (Kersten et al. 1985), because the very similar $PmdAB_{BR6020}$ (see below) presumably dechlorinates the compound during degradation of 3-chlorobenzoate (Nakatsu et al. 1995).

Sequence of *pmdAB* from *C. testosteroni* T-2

The identical N-terminal amino acid sequences of PmdA and PmdB in strains T-2 and BR6020 (see above) implied high levels of sequence conservation. The corresponding genes are of identical length in each organism, 450 nucleotides for *pmdA* and 870 nucleotides for *pmdB*, with sequence identities of 91% and 88%, respectively. There was 99% identity in the appropriate amino acid sequences (149 and 289 amino acids).

Gene sequences ($pmdAB_{T\text{-}2}$, $pmdAB_{BR6020}$, *proOab*, *pcmA*, *ligAB*, *fldVU* and *carBab*) were subjected to a statistical Markov analysis to estimate the phylogenetic branch points during evolution (Saccone et al. 1990, 1993). Similar analysis of *xylE* (Acc. M64747) and *nahH* (Acc. M17159), which encode class I enzymes, indicate 30 million years of independent evolution (Harayama and Rekik 1993; Hirose et al. 1994). Divergent evolution within the named members of the class III genes was apparently initiated more than 60 million years ago. The same analyses indicate that $pmdB_{T\text{-}2}$ and $pmbB_{BR6020}$ have been diverging for about 5 million years, similar to $pmdA_{T\text{-}2}$ and $pmbA_{BR6020}$ (4 million years).

Discussion

The specific growth rate of *C. testosteroni* T-2 with PCA as a substrate (0.31 h/h) and the molar growth yield (36 g protein/mol PCA) (Fig. 2) allow a minimum specific activity for the ring-cleavage enzyme to be calculated, 2.4 mkat/kg protein. The specific activity measured in whole cells (Fig. 2) and in cell extracts (Tables 1, 2) is higher than this minimum, so it is likely that we have isolated the correct enzyme. The structure of the product of the reaction is routinely presented in the *E,E* conformation (Fig. 1), but it is presumably present in several conformations (Fig. 1), as are intermediates in the extradiol cleavage pathway of catechol, where the open-chain conformation dominates (e.g. Junker et al. 1994a). The presence of the bulky carboxylate in HCMS probably reduces the proportion of the molecules in the *Z,Z* confirmation and metabolism flows through the *Z,E* confirmation. This may explain why HCMS so readily undergoes the ring-closure reaction with ammonia (via the *Z,E* conformation), whereas the open-chain pathway product from catechol reacts slowly (Dagley et al. 1960).

Sequence data confirm that enzymes very similar to PmdAB occur in *Sphingomonas* spp. (Noda et al. 1990; Sugimoto et al. 1999), *Pseudomonas straminea* (formerly *P. ochraceae*) (Maruyama et al. 2004), *Pseudomonas* sp. K82 (Yun et al. 2004), *Rhodopseudomonas palustris* (Larimer et al. 2004) and in the Gram-positive *Arthrobacter keyseri* (Eaton 2001) (see below). Based on amino acid compositions, we believe that the enzyme from *C. testosteroni* Pt-L5 (Arciero et al. 1983), from which a definitive picture of the enzyme emerged, is also very similar to PmdAB. However, the similarity did not allow the purification protocol for strain Pt-L5 to be transferred to strain T-2. Likewise, the protocol for strain T-2 was only partially effective for strain BR6020, where only three differences at the amino acid level were detected (D46N and K146A in PmdA, V52I in PmdB). In other systems, a single exchanged amino acid alters the stability of an enzyme (Fersht 1999) or suffices for a PCA 3,4-dioxygenase to cleave 4-sulfocatechol (Contzen et al. 2001), and minor changes in the sequence of dichloromethane dehalogenase alter the K_m, K_{cat} and pH optimum (Vuilleumier et al. 2001). We anticipate much microheterogeneity in the PmdAB genes of other strains, given the differences uncovered in this study between *C. testosteroni* T-2 and BR6020 (see above). We believe further that *C. testosteroni* codes for only one Pmd. The same enzyme was present in all the growth conditions we examined in strain T-2 (Table 1). Furthermore, in Southern blot analysis with four strains of *C. testosteroni*, only one band hybridized (M.A. Providenti, unpublished data), and a knockout of *pmdA* in strain BR6020 confirmed the absence of an enzyme which could substitute for it (Providenti et al. 2001).

We believed that the specific activity of PmdAB in *C. testosteroni* T-2 was constant during and after growth (Schläfli Oppenberg et al. 1995), which was largely the case when, e.g. *p*-toluenesulfonate was the growth substrate (Mampel 2000). More frequent sampling during growth in PCA-salts medium, however, showed that the level fluctuates markedly (Fig. 2). We wonder if a similar phenomenon is found in other strains of *C. testosteroni*, because Arciero et al. specify precisely when to harvest cells for optimal enzyme purification (Arciero et al. 1983). The phenomenon of the spike of activity was also detected with *p*-toluenesulfonate dioxygenase and *p*-sulfobenzoate dioxygenase (Mampel 2000), where analyses of levels of mRNA encoding these dioxygenases indicated no increase in messenger during the spike. Moreover, mRNA encoding *p*-toluenesulfonate monooxygenase, *p*-sulfobenzoate dioxygenase and PmdAB was not detected in cells with significant activity in the

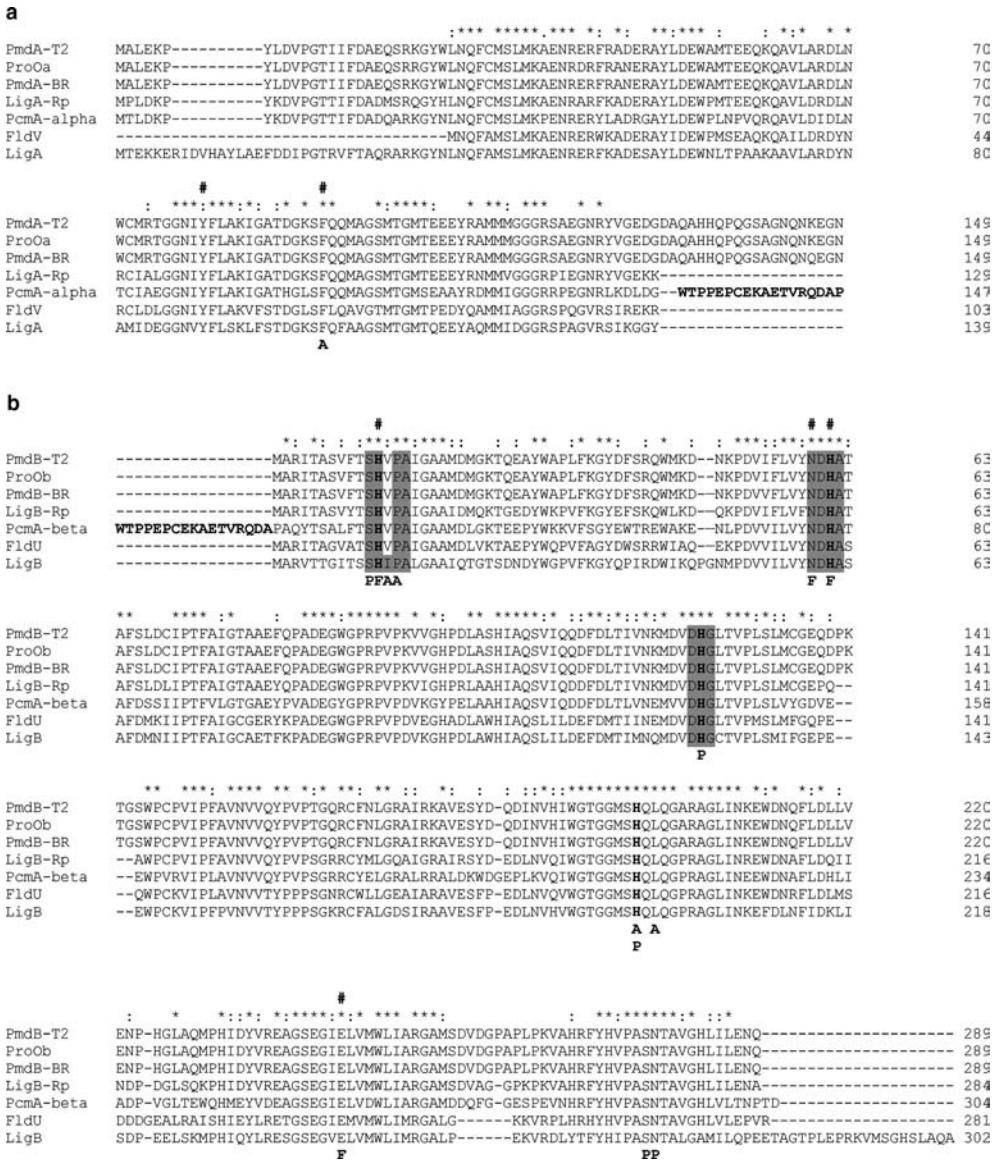

Fig. 4 Alignments (Clustal X) of the α subunits (**a**) and of the β subunits (**b**) of PmdAB from *C. testosteroni* T-2 with extradiol dioxygenases of > 60% sequence identity. PcmA is a monomer, but was considered as two subunits for sequence comparisons; the linker sequence is shown in *boldface* at the C terminus of the α subunit and at the N terminus of the β subunit. Histidine residues in *boldface* are conserved in all class III extradiol oxygenases. Signature sequences for iron ligands observed in crystals of LigAB are indicated by *grey boxes* within the sequence. Amino acid residues, which interact with the PCA molecule or the mononuclear iron in LigAB, are marked by *letters under the sequence*: *A* nonspecific hydrophobic interaction with the aromatic ring of PCA, *F* coordination of the iron-atom, *P* specific interaction with hydroxyl or carboxyl moieties of PCA, *asterisks* positions with a conserved residue, *colons* positions with a conservative replacement. In **a**, *crosshatches* represent amino acids of the α subunit involved in catalysis; in **b**, *crosshatches* represent iron ligands identified for LigB. Data used: PmdAB-T2, (this study) PmdAB-BR (AF305325, Providenti et al. 2001); ProOab, from *Pseudomonas ochraceae* (AB127969, Maruyama et al. 2004); PcmA, from *Arthrobacter keyseri* 12B (AF331043, Eaton 2001); FldVU, putative protein from *Sphingomonas* sp. LB126 (AJ277295, Wattiau et al. 2001); Lig AB, from *S. paucimobilis* SYK-6 (M34835, Noda et al. 1990); LigAB-Rp, from *Rhodopseudomonas palustris* CGA009 (LigA: NP_950035; LigB: NP_950036, Larimer et al. 2004)

stationary phase. We thus presume that the overall level of oxygenase activity in this organism is controlled not only by transcriptional but also—and presumably more substantially—by post-translational processes.

The deduced amino acid sequences of each subunit of $PmdAB_{T\text{-}2}$ show high levels of identity to annotated extradiol dioxygenases with an $\alpha\beta$-subunit structure. After ProOab and $PmdAB_{BR6020}$ (98–100% identity to the domains corresponding to the α and β subunits, respectively, of PmdAB) and LigAB from *Rhodopseudomonas palustris* (81–84%), the highest identity is to monomeric PcmA of *A. keyseri* 12B (71 and 66% identity), followed by LigAB of *S. paucimobilis* SYK-6 (67 and 60%) and FldVU, a putative PmdAB of *Sphingomonas* sp. LB126 (66 and 65%). There was a clear cutoff to other sequences in databases (65%); the

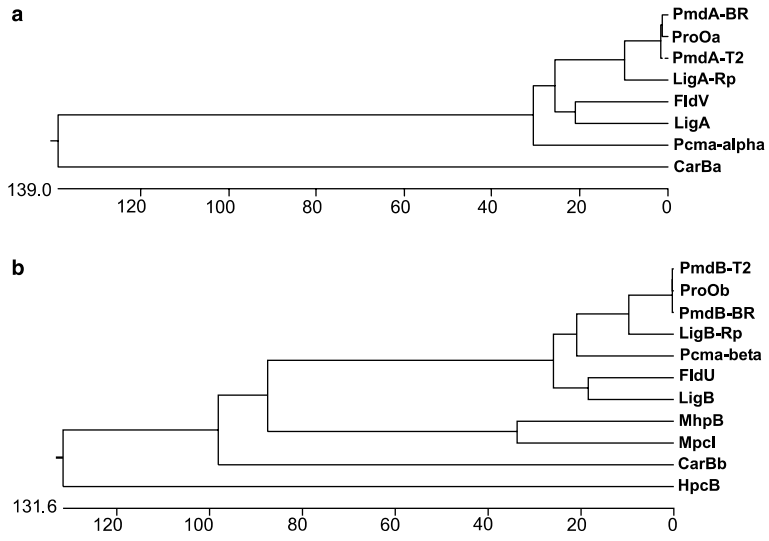

Fig. 5 Balanced dendrograms of α subunits of heteromeric class III extradiol oxygenases (**a**) or β subunits of class III extradiol oxygenases (**b**). Complete amino acid sequences were aligned using the Clustal method of the DNAStar program Megalign prior to generating the dendrogram; the PAM250 residue weight table was used. The length of the branches indicate relative phylogenetic distances between the individual sequences, so related oxygenases are clustered (accession numbers for sequences are given in *brackets*): *PmdAB*, *C. testosteroni* T-2 or BR6020, PmdAB (AF459635, AF305325); *ProOab*, *P. ochraceae* PCA 4,5-dioxygenase (PCA-DO) (AB127969); *PcmA*, *A. keyseri* 12B, PCA-DO (AJ277295); *FldUV*, *Sphingomonas* sp. strain LB 126, putative PCA-DO, (AJ277295); *LigAB*, *S. paucimobilis* SYK-6, PCA-DO; *LigAB-Rp*, *R. palustris* CGA009, PCA-DO; *CarBab*, *Pseudomonas* sp. CA10, 2-aminobiphenyl 2,3-diol-1,2-dioxygenase (D89064); *MhpB*, *Escherichia coli*, 2,3-dihydroxyphenylpropionate dioxygenase (D86239); *MpcI*, *Ralstonia eutrophus* JMP222, 2,3-dihydroxyphenylpropionate dioxygenase (X52414); *HpcB*, *E. coli*, homoprotocatechuate dioxygenase (Q05353)

next highest level of identity (28%) is for CarBab, 2-aminobiphenyl-2,3-diol-1,2-dioxygenase of *Pseudomonas* sp. CA10 (Sato et al. 1997).

Sequence alignments with the corresponding subunit showed extensive sequence identity in the core of PmdA and over most of PmdB, and allowed key common features to be identified (Fig. 4). FldV, the smallest protein, appears to comprise just the core sequence of the α subunit, which includes two residues (Y90 and F103 in PmdA) corresponding to interactions with substrate in LigA (Fig. 4). Four conserved histidine residues (H12, H59, H125 and H197 in $PmdB_{T-2}$) in the β subunit are relevant, as is an asparagine (N57 in $PmdB_{T-2}$). H12, N57 and H59 correspond to the iron-binding residues in the LigB-structure, while H125 and H197 correspond to residues that interact with polar and nonpolar regions of the aromatic substrate in LigB (Fig. 4). These histidines are found in four known motifs with the asparagine: SHXPA (H12 in PmdB) and NDHA (H59 in PmdB) in iron coordination, and DHG (H125 in PmdB) and GXSH (H197 in PmdB). These motifs are characteristic of the class III extradiol dioxygenases (Eltis and Bolin 1996; Peng et al. 1998). The consensus motif derived from extradiol dioxygenases of classes I and II (PROSITE PS00082) (Eltis and Bolin 1996) is absent.

A dendrogram (Fig. 5a), derived from the sequences of the α subunits of effectively heteromultimeric extradiol dioxygenases, shows that the seven sequences (Fig. 4) cluster together, but show some relationship to CarBa. A dendrogram of the β subunits of all known representatives of extradiol oxygenases in class III shows four major lines, two of which are known by only one representative, HpcB and CarBb. A third group has at least two representatives, MhpB and MpcI, whereas the fourth and currently largest group is that of the β subunits of effectively heteromultimeric extradiol dioxygenases (Fig. 5b). The tight grouping of these β subunits corresponds to the dendrogram for the α subunits (Fig. 5a). Class II oxygenases acting on monocyclic substrates diverge from those acting on biphenylic substrates (e.g. BphC-LB400). We wonder whether this subgrouping according to substrate (CarBab and LigAB) is present in class III.

Acknowledgements We are grateful to T. Tralau, who kindly did the Markov analyses, and to D. Schleheck, who did the lutidinic acid experiment. J.M. was funded by the Deutsche Forschungsgemeinschaft (to A.M.C. and J. Ruff) and BASF/BMBF, and M.A.P. by the Alexander von Humboldt Stiftung. Further funds were from the University of Konstanz and the Fonds der Chemischen Industrie.

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