Biodegradation (2010) 21:267–281
DOI 10.1007/s10532-009-9299-2

ORIGINAL PAPER

Conversion of polycyclic aromatic hydrocarbons, methyl naphthalenes and dibenzofuran by two fungal peroxygenases

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Received: 15 July 2009 / Accepted: 9 September 2009 / Published online: 22 September 2009

Abstract The aim of this work has been to study the substrate specificity of two aromatic peroxygenases concerning polyaromatic compounds of different size and structure as well as to identify the key metabolites of their oxidation. Thus, we report here on new pathways and reactions for 2-methylnaphthalene, 1-methylnaphthalene, dibenzofuran, fluorene, phenanthrene, anthracene and pyrene catalyzed by peroxygenases from *Agrocybe aegerita* and *Coprinellus radians* (abbreviated as AaP and CrP). AaP hydroxylated the aromatic rings of all substrates tested at different positions, whereas CrP showed a limited capacity for aromatic ring-hydroxylation and did not hydroxylate phenanthrene but preferably oxygenated fluorene at the non-aromatic C_9-carbon and methylnaphthalenes at the side chain. The results demonstrate for the first time the broad substrate specificity of fungal peroxygenases for polyaromatic compounds, and they are discussed in terms of their biocatalytic and environmental implications.

Keywords Basidiomycete · Peroxidase · Heterocyclic compounds · Dibenzofuran · Hydroxylation · Oxygenation

Introduction

The development of clean biocatalytic methods for the conversion of aromatic compounds has received increasing attention over the last two decades both against the background of organic synthesis ("Green" chemistry) and natural attenuation processes. As it is well known, polycyclic aromatic hydrocarbons (PAHs) and related compounds are recalcitrant organopollutants occurring in crude oil, coal and their refined products. Thus, emissions from petroleum refining, coal tar distillation as well as gasoline and diesel fueled engines are the major contributors of PAHs to the environment (Baek et al. 1991; Harvey 1991). In addition, methylnaphthalenes are precursors in the production of pharmaceuticals and pesticides and hence are released via manufacturing effluents (Murata et al. 1993). In this context, biocatalytic oxygen transfer reactions are of particular importance since they can selectively change the chemical properties of PAHs and thereby their ecotoxicological and physiological effects. Extracellular fungal oxidoreductases would represent a promising tool in catalyzing such oxidations since they are stable and just need simple co-substrates (Torres et al. 2003; Ullrich and Hofrichter 2007).

Electronic supplementary material The online version of this article (doi:10.1007/s10532-009-9299-2) contains supplementary material, which is available to authorized users.

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Over the recent years, we have studied a new type of peroxide-consuming enzyme with a great potential for this purpose, the aromatic peroxygenase (APO). This designation has recently been coined because of the enzyme's ability to catalyze the epoxidation, hydroxylation and hetero-atom oxygenation of aromatic and heterocyclic rings. For example, APO of *Agrocybe aegerita* is able to perform the sulfoxidation and hydroxylation of dibenzothiophene (Aranda et al. 2009), the ring and side-chain hydroxylation of toluene (Ullrich and Hofrichter 2005), the *N*-oxidation of pyridine (Ullrich et al. 2008) as well as the selective epoxidation of naphthalene (Kluge et al. 2009). APO enzymes are secreted heme-thiolate proteins which have been described in detail just for two agaric basidiomycetes, *A. aegerita* and *Coprinellus radians* colonizing wood, mulch and dung (Ullrich et al. 2004; Anh et al. 2007), but there are molecular indications for their wide-spread occurrence in the fungal kingdom and even beyond that (Pecyna et al. 2009).

Though a number of aromatic compounds were found to be substrates of APO and a plausible catalytic mechanism has been postulated (Ullrich and Hofrichter 2007), the spectrum of oxidizable substrates regarding molecule size and structural arrangement is still not clear. Therefore, we compare here the oxidation of different polycyclic aromatic hydrocarbons (PAHs) and related molecules (methylnaphthalenes, dibenzofuran) by APO of *A. aegerita* (AaP) and *C. radians* (CrP). Due to the catalytic similarity of extracellular APO to intracellular cytochrome P450 enzymes—representing the most important group of aromatic monooxygenases (Guengerich 1991)—our results may be both of environmental and biotechnological relevance. Not least, this becomes evident by the fact that fungal peroxidases including APO-like enzymes are actively secreted by fungi into their microhabitats and hence to different environmental compartments such as soil, leaf-litter or wood.

Materials and methods

Enzyme preparation

Production of the major peroxygenase forms AaP II from *Agrocybe aegerita* (TMA1; DSMZ 22459) and CrP II from *Coprinellus radians* (DSMZ 888) was carried out in 10-l stirred tank bioreactors and agitated flasks (500 ml), respectively. The growth media used were 2% soybean flour for *A. aegerita* and 3% soybean flour supplemented with 4% glucose for *C. radians*. The cell-free medium obtained was concentrated by ultrafiltration and the enzymes were purified by several steps of ion exchange chromatography and size exclusion chromatography according to Ullrich et al. (2004) and Anh et al. (2007). SDS–PAGE was performed to prove the purity of the enzyme preparations (data not shown); they had final specific activities of 62 U mg^{-1} (AaP) and 35 U mg^{-1} (CrP) using veratryl alcohol as substrate (Ullrich et al. 2004).

Chemicals

Following PAHs and related compounds were tested as APO substrates: fluorene (Flu), anthracene (Ant), phenanthrene (Phe), pyrene (Pyr) and perylene (Per), 2-methylnaphthalene (2-MN) and 1-methylnaphthalene (1-MN) as well as the heterocycle dibenzofuran (Dbf). They were purchased along with potential oxidation products (1-naphthoic acid, 2-naphthoic acid, 1-naphthaldehyde, 2-naphthaldehyde, 1-naphthalene methanol, 2-naphthalene methanol, 9-fluorenol, 9-fluorenone, 2-hydroxyfluorene, 1,9-anthraquinone, 1-anthrone, 9,10-phenanthroquinone, 1-hydroxypyrene) as well as the derivatization agent 2,4-dinitrophenylhydrazine (DNPH) as fine chemicals from Sigma–Aldrich (Steinheim, Germany). HPLC grade acetonitrile, Tween 20, ascorbic acid, and H_2O_2 (30%, w/v) were obtained from Merck (Darmstadt, Germany). All PAH stock solutions and standards were prepared in acetonitrile. DNPH stock solution (0.1%) was obtained by dissolving 100 mg of DNPH in 50 ml of boiling water with 5 ml of concentrated HCl. The solution was diluted with water to 100 ml and was stored at 3°C in the dark (Nassar et al. 2004).

Enzymatic reactions

Enzymatic reactions were carried out in 1.5-ml HPLC vials. Reaction mixtures (1 ml) containing 50 mM potassium phosphate buffer (pH 7), 20% acetonitrile, 1 mM of each PAH, 1 Unit of AaP or CrP (defined as veratryl alcohol oxidation units, equivalent to 0.36 μM) were incubated at room temperature (22°C) over 15 min. Parallel experiments were carried out in the presence of ascorbic acid (5 mM final

concentration) in order to prevent further oxidation of phenolic products formed (Osman et al. 1996). Control experiments without enzymes or with boiled enzymes were performed but no relevant PAH conversion could be observed. Reactions were started by the addition of hydrogen peroxide. It was continuously supplied with a syringe pump at a flow rate of 160 µl h^{-1} (final H_2O_2 concentration, 5 mM) and the reaction mixtures were kept under permanent stirring by a magnet. After preliminary tests, the reaction time for the oxidation of larger PAHs (Phe, Ant, Pyr and Per) was increased up to 8 h due to the slowness of the reaction. In these experiments, 1% Tween 20 was added to improve the solubility of the substrates. Samples (30 µl) were collected every hour and to keep the total volume constant, the same amount of diluted H_2O_2 (30 µl) was added with the syringe pump to the reaction mixture. The reaction was stopped by addition of acetonitrile (final concentration 75% v/v). In the case of 1-MN, 2-MN, Dbf and Flu, the reaction was stopped with 0.15 ml of 50% w/v trichloroacetic acid and the samples were centrifuged (1,000*g*) for 3 min. Supernatants (5 µl) were directly injected into an HPLC system; for metabolite identification, 30 µl of the reaction solution was used. In all cases, three replicates were analyzed.

Chemical analyses and product identification

Substrate conversion and metabolites were analyzed using an HPLC system HP 1100 (Agilent®, Waldbronn, Germany) equipped with a diode array detector (DAD; 190–700 nm) and an Agilent MSD-VL mass spectrometer system (API-ES). Separations were carried out on a reversed phase column, Synergy Fusion RP C18 80A (4 µm, 4.6 × 125 mm; Phenomenex®, Aschaffenburg, Germany). Elution solvents were 15 mM phosphoric acid in water (A) and acetonitrile (B). The temperature of the column was set to 50°C. Metabolites were separated with a constant flow rate of 1 ml min^{-1} in a linear gradient elution mode starting with 30% B, reaching 80% B within 10 min and being maintained at 80% during 3 min until all analytes were eluted from the column. In the case of 2-MN and 1-MN, the elution started with 5% B and the gradient was as follows: 0–3.5 min, 5% B; 3.5–10 min, 15% B; 10–13 min, 26% B; 13–16 min, 30% B; 16–36 min, 80% B and 37–41 min, 5% B.

LC/MS analyses were performed under the same chromatographic conditions using acetonitrile and 0.01% v/v formic acid (HCOOH) in an ammonium buffer (pH 3.5) as mobile phase. Ionization was achieved using atmospheric pressure electrospray ionization (API-ES). The eluting compounds were ionized in the positive or negative mode. Better resolution was achieved for 2-MN and 1-MN using a reversed phase column, Luna C18 100A (5 µm, 150 × 2.00 mm; Phenomenex®, Aschaffenburg, Germany). The flow rate was set at 0.35 ml min^{-1} and elution conditions were the same as described above.

Oxidation products were identified by comparing the retention times, mass spectra and UV-spectra with authentic standards (when available) and with literature data. In absence of reference data, metabolites were tentatively indentified according to their mass spectra.

Derivatization

A hydrazine derivatization reagent (2,4-dinitrophenylhydrazine, DNPH) was used for the detection of aldehydes formed in the course of 2-MN and 1-MN conversion. Derivatization of the samples was achieved by adding 1% of the reagent solution (0.1% DNPH) directly to the reaction mixtures at room temperature. DNPH derivatives (the corresponding hydrazones) were detected at 360 nm and identified by their mass spectra under the chromatographic conditions given above.

Results

Conversion of 2-methylnaphthalene (2-MN) and 1-methylnaphthalene (1-MN)

2-MN was rapidly and completely converted by both peroxygenases (AaP, CrP) and a variety of oxidation products (up to 14!) were detectable. The main metabolites were 2-naphthoic acid (AaP and CrP) and *mono*-hydroxylated 2-MNs (*mono*-OH-2-MN; Table 1). The former compound and its precursors: 2-naphthalene methanol and 2-naphthaldehyde were unambiguously identified by comparing their analytical data with authentic standards. In addition, their corresponding ring-hydroxylated metabolites

Table 1 Retention times, UV-spectral characteristics, and API-ES mass spectrometric data of selected metabolites produced as the results of the treatment of methylnaphthalenes (MN), dibenzofuran (Dbf) and several PAHs with fungal aromatic peroxygenases

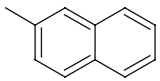
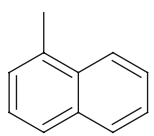
Substrate	Metabolites formed	Rt	*m/z* $[M-H]^-$	UV λ (% relative intensity)
2-MN 	*mono*-OH-2-MN (a)	23.124	157	234(100) 214(94) 296(11) 327(5)
	mono-OH-2-MN[4] (b)	22.445	157	227(100) 254(4) 263(6) 273(6) 283(4) 335(3)
	mono-OH-2-MN(c)	22.985	157	228(100) 250(96) 283(25) 291(23) 327(6) 346(5)
	2-naphthaldehyde	22.894	(•)	250(100) 284(22) 292(21) 344(5)
	2-naphthoic acid	20.930	171	237(100) 271(10) 281(13) 289(9) 322(2) 334(3)
	2-naphthalene-methanol[1,4]	19.133	(•)	224(100) 267(5) 274(5) 285s(4)
	mono-OH-2-naphthaldehyde (a)	19.833	171	259(100) 221(76) 293(20) 363(15)
	mono-OH-2-naphthaldehyde (b)	19.097	171	260(100) 216(62) 298(12) 368(14)
	mono-OH-2-naphthaldehyde (c)	17.838	171	260(100) 212(62) 291(13) 365(10)
	mono-OH-2-naphthoic acid (a)	17.631	187	248(100) 213(64) 288(9) 344(7)
	mono-OH-2-naphthoic acid (b)	16.526	187	247(100) 213(67) 297(8) 344(10)
	mono-OH-2-napthalenemethanol (a)	16.398	173	227(100) 265(6) 275(6) 287(4) 328(3)
	mono-OH-2-napthalenemethanol (b)	16.291	173	215(100) 233(98) 293(12) 312(8) 325(6)
	di-OH-2-naphthalenemethanol[1,3]	13.615	189	217(100) 240(69) 287(9) 331(5)
1-MN 	*mono*-OH-1-MN[4] (a)	23.814	157	224(100) 300(14) 316(11) 329(10)
	1-naphthaldehyde[4]	23.096	(•)	211(100) 243(40) 317(19)
	mono-OH-1-MN (b)	22.755	157	223(100) 298(15) 311(11) 325(7)
	mono-OH-1-MN (c)	22.663	157	210(100) 237(76) 303(14) 327(9)
	mono-OH-1-MN (d)	22.337	157	229(100) 269(6) 279(7) 290(6) 332(3)
	mono-OH-1-MN[3] (e)	22.125	157	227(100) 309(14) 324(13) 338(13)
	mono-OH-1-MN[1] (f)	22.021	157	227(100) 270(7) 279(9) 291(8) 332(4)
	1-naphthoic acid	20.002	171	218(100) 211s(91) 232(s) 295(15)
	di-OH-1-MN[1,3] (a)	19.479	173	233(100) 217(56) 288(9) 336(6)
	1-naphthalene-methanol[4]	18.617	(•)	223(100) 271(7) 281(9) 291(6)
	di-OH-1-MN[3] (b)	17.157	173	285(100) 242(96)
	di-OH-1-MN[1,3] (c)	16.511	173	229(100) 215(91) 305(24)
	mono-OH-1-naphthoic acid[3] (a)	15.451	187	208(100) 245(54) 278(17)
	mono-OH-1-naphthoic acid[1] (b)	15.537	187	229(100) 264(6) 274(6) 285(4) 346(4)
	mono-OH-1-naphthoic acid[1] (c)	14.676	187	228(100) 208(81) 274(37)
	mono-OH-1-naphthalenemethanol[1,3]	13.740	173	215(100) 231(87) 297(17)
	mono-OH-1-naphthoic acid[1] (d)	13.340	187	232(100) 273(7) 282(7) 293(5)

Table 1 continued

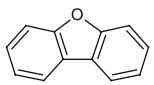
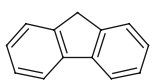
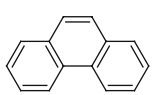
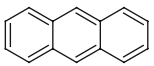
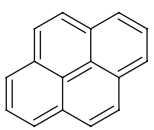
Substrate	Metabolites formed	Rt	*m/z* $[M-H]^-$	UV λ (% relative intensity)
Dbf	3-OH-Dbf	11.676	183	214(100) 231(47) 254(38) 298(47) 305(41)
	*mono*-OH-Dbf	11.332	183	212(100) 253(38) 280(35) 289(29)
	2,3 *di*-OH-Dbf	9.366	199	198(100) 216(90) 246(42) 299(38) 314(40)
	di-OH-Dbf (a)	8.621	199	215(100) 258(31) 289(38)
	di-OH-Dbf[1] (b)	8.225	199	226(100) 260(39) 270(33) 276(26) 297(10) 309(11)
	di-OH-Dbf (c)	7.492	199	223(100) 262(42) 283(30) 310(31)
	di-OH-Dbf (d)	6.851	199	222(100) 259(46) 288(37) 296(36) 307(27)
	3,7 di-OH-Dbf	6.236	199	218(100) 237(45) 255(39) 310(46)
	di-OH-Dbf (e)	5.862	199	216(100) 241(42) 257(47) 306(53)
	di-OH-Dbf[1] (f)	5.427	199	216(100) 254(27) 261(28) 288(29) 312(16) 323(18)
	tri-OH-Dbf[1]	3.907	215	219(100) 250(55) 313(49)
Flu	9-Fluorenone[4]	12.719	181*	257(100) 249(58) 296(4)
	2-OH-Flu	11.578	181	227(100) 220(91) 235(85) 271(58)
	Dimer[2,3] (a)	10.724	393	278(100) 257(76) 332(36)
	Dimer[2,3] (b)	9.944	393	276(100) 266(89) 326(33)
	9-Fluorenol[4]	9.787	181	271(100) 305(30) 312(30)
	di-OH-Flu[2,3] (a)	8.762	197	272(100) 290(51) 319(91)
	di-OH-Flu (b)	5.310	197	274(100) 319(72)
	mono-OH-Fluorenone[2,3]	5.802	197*	247(100) 340(60)
	tri-OH-Flu[1,3]	3.752	213	276(100) 295(56) 328(61)
Phe	4-phenanthrol[3]	12.936	193	245(100) 213(55) 226(51) 274(43) 302(18) 338(6) 355(6)
	1-phenanthrol[3]	12.568	193	251(100) 247(99) 258(92) 223(83) 295(25) 305(26) 338(7) 356(7)
	3-phenanthrol[3]	12.108	193	252(100) 221(49) 277(29) 291(17) 298(16) 339(3) 357(3)
	di-OH-Phe[3] (a)	10.823	209	223(100) 264(97) 298s(19) 346(4) 363(5)
	di-OH-Phe[3] (b)	10.495	209	234(100) 291(88) 380(12)
	di-OH-Phe[1,3] (c)	9.503	209	247(100) 213(78) 285(57) 305(27) 316(27) 342(4) 360(3)
	di-OH-Phe[3] (d)	9.127	209	215(100) 284(64) 305(38) 317(38) 342(7) 359(6)
	di-OH-Phe[3] (d)	8.011	209	258(100) 236 (56) 297(22) 309(25) 345(8) 364(9)
	Unknown peak[1,3]	6.200	223	219(100) 235s(92) 304(65)
	Unknown peak[1,3]	5.416	223	247(100) 216(80) 310(58)
	Unknown peak[3]	4.693	(•)	267(100) 241(74) 298(22) 310(12)
	Unknown peak[1,3]	3.521	(•)	278(100) 242(67) 233(56) 271(95) 308(24) 320(25)

Table 1 continued

Substrate	Metabolites formed	Rt	*m/z* $[M-H]^-$	UV λ (% relative intensity)
Ant	*mono*-OH-Ant (a)	12.321	193	254(100) 237(43) 373(5)
	*mono*-OH-Ant (b)	12.105	193	255(100) 331(3) 352(3) 375(3) 396(3)
	di-OH-Ant (a)	10.082	209	258(100) 366(5) 384(5)
	Unknown[1,4]	9.348	265	260(100) 243(62) 370(9)
	Unknown (putative quinone)	9.069	223	258(100) 348(3) 350(3) 362(2) 381(5)
	di-OH-Ant (b)	8.591	209	258(100) 366(5) 381(5) 399(4) 402(4)
	Unknown[1,3](OH-Anthraquinone)	6.105	223	248(100) 301(95)
	Unknown[1,4]	6.500	(•)	239(100) 215(80) 303(9) 329(7)
Pyr	1-Pyrenol[1,2,3,4]	13.261	217	241(100) 268(33) 278(57) 347(38) 365(17) 385(12)
	1,8 *di*-OH-Pyr[1–3,2–3,2–4]	10.195	232	241(100) 271(35)s 281(47) 353(42) 394(8)
	1,6 *di*-OH-Pyr[1,3]	9.755	232	245(100) 237(64) 267(36) 278(63) 336(34) 351(48) 379(16) 399(20)
	Unknown	5.981	(•)	241(100) 273(52) 352(33) 369(43) 385(75)

[1] Only in presence of ascorbic acid, [2] Only in absence of ascorbic acid, [3] Only produced by AaP, [4] Only produced by CrP, (•) not detectable by APIES-MS, * not detectable in negative mode, data corresponding to the obtained value in positive mode $[M+H]^+$

(hydroxy-2-napththoic acid, hydroxynaphthaldehyde and hydroxynaphthalene methanol) were tentatively identified by their mass spectra. The high amount of naphthoic acid obtained in both reactions indicates that the oxidative attack on 2-MN preferably occurs at the methyl group, though there were slight differences between AaP and CrP. Thus in the absence of ascorbic acid, the yield of 2-naphthoic acid was 105 and 80 μM for CrP and AaP, respectively. Minor amounts of other hydroxylation products (e.g. *di*-OH-naphthalene methanol) were also observed but just in case of AaP and when ascorbic acid was present. CrP showed only a limited capacity for aromatic ring-hydroxylation (production of traces of *mono*-OH-2-MN) and the oxygenation of the methyl group by CrP was the favorite mode of action, whereas AaP likewise followed both pathways (i.e. ring and side-chain oxidation).

When 1-MN was used as substrate, the main metabolites formed by AaP were *mono*-OH-1-MN and *di*-OH-1-MN, which differs from the pronounced side-chain oxidation in case of 2-MN (supplementary material Fig. S1) and indicates a preference for ring-hydroxylation. Nevertheless, small amounts (<3 μM) of 1-naphthoic acid were also found in the reaction solution demonstrating the—albeit limited—oxidation of the side-chain up to the carboxylic end-product by AaP. Products were identified by means of authentic standards. Furthermore, traces of a hydroxylated 1-naphthoic acid were formed by both APOs. 1-MN was not completely converted by CrP (76%) and the quantification of 1-naphthoic acid yielded 44 μM. In contrast, the complete oxidation of 1-MN was accomplished by AaP but only traces of the corresponding acid (3 μM) were formed. This shows again the preferred side-chain oxidation by CrP and the favored ring hydroxylation by AaP.

Derivatization of the reaction solutions (2-MN, 1-MN) with DNPH was performed in order to distinguish between differently hydroxylated naphthaldehydes, which are not available as standards (the respective dinitrophenylhydrazones are adducts of DNPH and the aldehyde group). Analysis by LC–MS revealed the presence of three different *mono*-OH-2-naphthaldehydes with a base peak that shifted from *m/z* 172 to *m/z* 352 in the case of AaP; only negligible amounts of one *mono*-OH-2-naphthaldehyde were detected in the presence of CrP (Table 1). In the case of 1-MN, no hydroxylated 1-naphthaldehyde was found suggesting the blocking of ring-hydroxylating activity when an aldehyde function is at the *alpha*-position in the naphthalene molecule.

The formation of hydroxylated naphthaldehydes and hydroxylated naphthoic acids was additionally proved, in both cases, by means of independent incubations with 1- and 2-naphthalene methanol, 1- and 2-naphthaldehyde as well as 1- and 2-naphthoic acid (data not shown). Interestingly, both peroxygenases were again not able to form a hydroxylated 1-naphthaldehyde, which corresponds with the previous observation during 2-MN oxidation.

In summary the results suggest that, after the initial oxygenation of 2-MN's methyl group, the product (2-naphthalene methanol) is stepwise oxidized into 2-naphthoic acid via 2-naphthaldehyde as intermediate. In case of 1-MN, a sterical hindrance of APO for side-chain oxidation can be proposed since aromatic hydroxylation is the favorite pathway (Fig. 1a, b). Two representative HPLC elution profiles recorded under different conditions illustrate MN conversion by AaP and CrP (supplementary material Fig. S1).

Dibenzofuran (Dbf) conversion

Both peroxygenases were capable of converting Dbf via hydroxylation at different positions. Thus in the presence of the radical scavenger ascorbic acid, AaP formed up to 11 different products, among which were *mono*-, *di*, and *tri*-hydroxylated metabolites (Fig. 2a). They were tentatively identified by their LC/MS data (retention times, UV spectra and mass spectra) (Table 1). The initial oxidation yielded two *mono*-OH-Dbf derivatives including 3-OH-Dbf (that was identified by comparison with literature data). Further hydroxylation occurred at both aromatic rings resulting in the formation of eight *di*-hydroxylated and one *tri*-hydroxylated metabolite (Fig. 2a). Up to five hydroxylated metabolites were found in CrP-containing samples in the presence of ascorbic acid (Table 1; Fig. 2b). Altogether, CrP was less efficient in Dbf oxidation than AaP was and converted only 43% of the compound (while AaP oxidized Dbf by 100%). 3-OH-Dbf, 2,3 *di*-hydroxy-Dbf, and 3,7 *di*-OH-Dbf were present in all samples and could be identified by comparing their spectra with literature data (Cerniglia et al. 1979; Hammer et al. 1998).

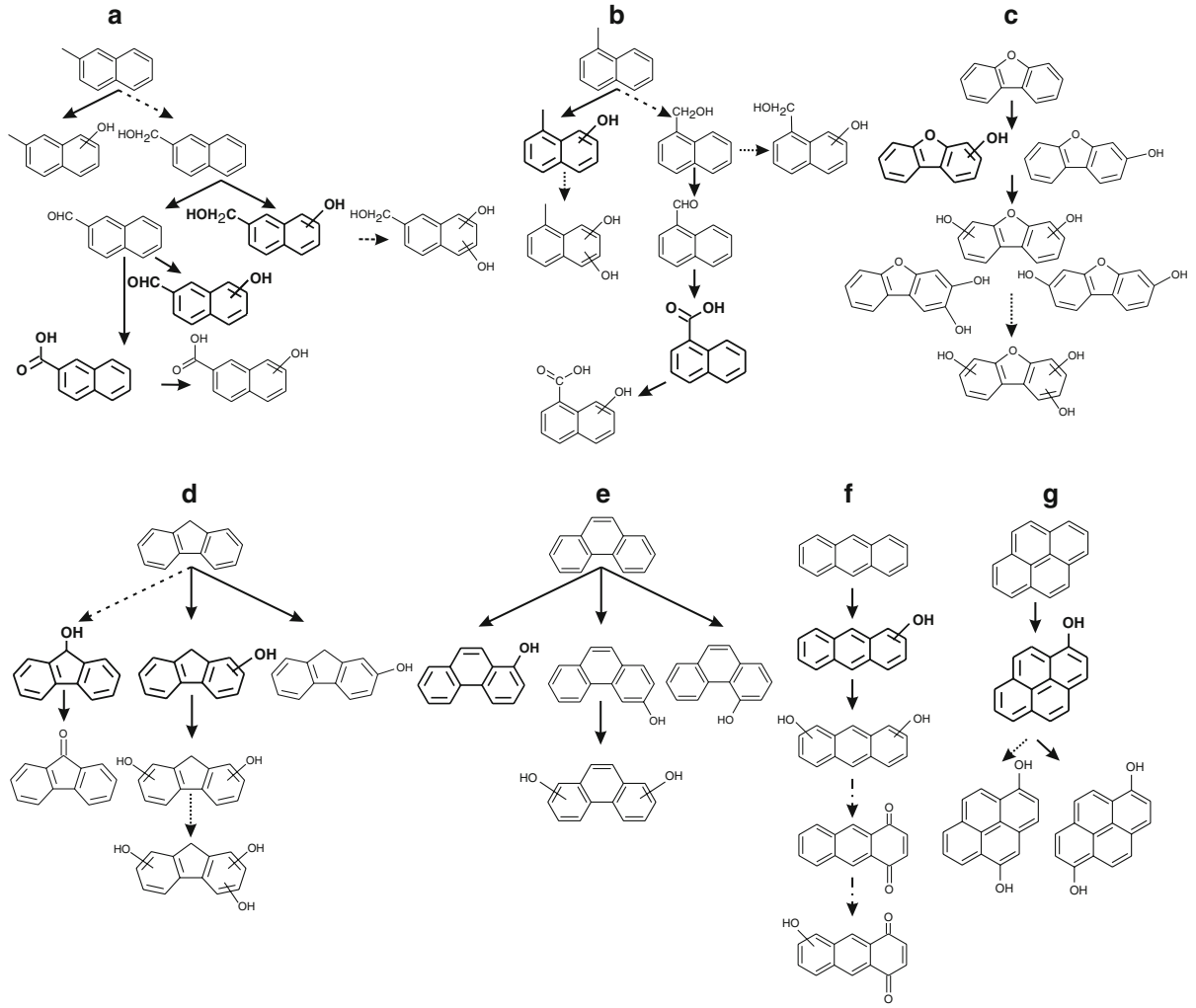


Fig. 1 Proposed pathways for the conversion of PAHs tested in this study by the peroxygenases of *A. aegerita* (AaP) and *C. radians* (CrP). 2-MN (**a**), 1-MN (**b**), Dbf (**c**), Flu (**d**), Phe (**e**), Ant (**f**) and Pyr (**g**). *Dashed arrows* indicate main pathways for conversion by CrP. *Dotted arrows* indicate the formation of metabolites solely observed in presence of the radical scavenger ascorbic acid. *Dashed-dotted arrows* indicate putative pathways according to the metabolites detected. The major metabolites of each pathway are given in *bold*

Fluorene (Flu) conversion

The major products identified after complete Flu conversion by AaP were 2-OH-Flu, *di-* and *tri-*hydroxylated metabolites, as well as two coupling products probably representing Flu dimers, which were formed when the radical scavenger was omitted (Table 1; Fig. 3a). We did not find evidence for the formation of 9-fluorenone and 9-OH-Flu (9-fluorenol) by AaP. Flu is usually easily oxidized at the C_9-position to form 9-fluorenone and even our controls without enzyme (but with H_2O_2) confirmed the formation of traces of this metabolite (<2 μM). This also explains the detection of a hydroxylated 9-fluorenone in the AaP reaction mixture, which may result from the hydroxylation of the autoxidation product 9-fluorenone (Fig. 3a). Additional experiments using 9-OH-Flu as substrate confirmed the inability of AaP to form 9-fluorenone (data not shown).

In contrast to AaP, CrP produced 9-OH-Flu and 9-fluorenone as the major metabolites (>5 μM), but

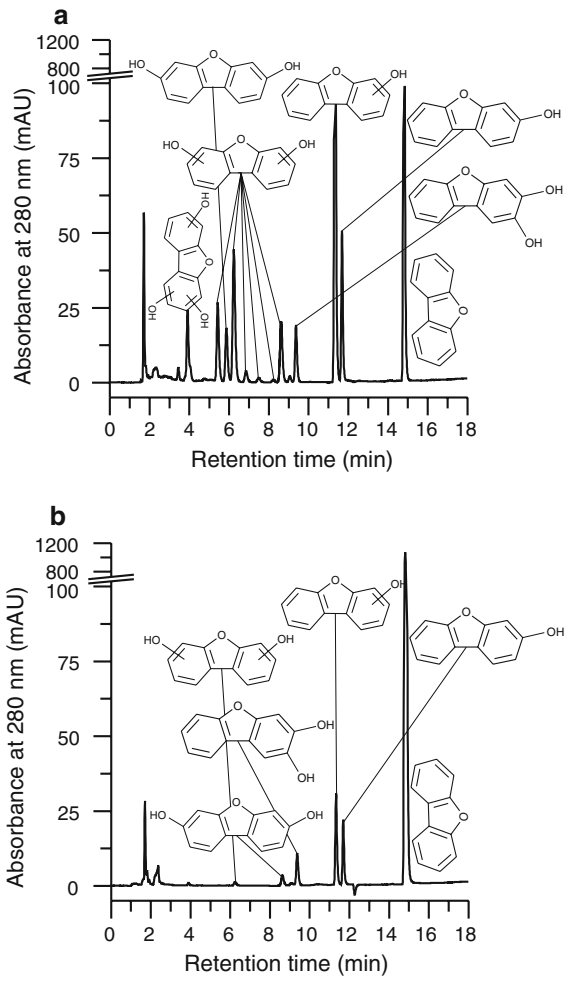


Fig. 2 HPLC elution profiles of metabolites formed during Dbf conversion by AaP (**a**) and CrP (**b**) in presence of ascorbic acid. Chromatograms were recorded at 280 nm

only in the absence of ascorbic acid and along with smaller amounts of 2-OH-Flu (<0.5 μM) (Fig. 3b). These metabolites were identified by means of authentic standards. In order to improve aromatic hydroxylation by CrP, the amount of enzyme was increased from 1 to 5 Units. As the result, the overall oxidation of Flu was noticeably enhanced (from 33 to 50%) and a *di*-OH-Flu was detected as additional metabolite (data not shown).

The results show that the main pathway of Flu conversion by CrP proceeds via the oxygenation/hydroxylation of the non-aromatic carbon at the 9-position while in the case of AaP, aromatic ring-hydroxylation is the major reaction (Fig. 1d).

Conversion of anthracene (Ant) and phenanthrene (Phe)

When increasing the size of the PAH-molecule to three aromatic rings, specific catalytic behaviors of peroxygenases were observed concerning the ring configuration. In the course of reaction, AaP almost completely converted Ant and Phe within 6 h, in absence of ascorbic acid (see supplementary material Fig. S2A; the start concentration of Ant/Phe was 1 mM, see "Materials and methods"). The initial rate of conversion was 186 and 275 μM h^{-1} for Ant and Phe, respectively (see supplementary material Fig. S2A). A lower rate of initial conversion (226 μM h^{-1}) for Phe was determined in the presence of ascorbic acid, but not in case of Ant (325 μM h^{-1}) due to its comparatively low redox potential (therefore it is oxidizable by most peroxidases and laccases; Hammel 1995; Johannes et al. 1996). In general, more hydroxylation products were detected under these conditions (Table 1). In all cases, the rates of Phe and Ant conversion by AaP slowed down in the further course of the experiments.

It is interesting to note that only negligible amounts of Phe (<5%) were oxidized by CrP within 8 h of reaction (both in the presence and absence of ascorbic acid; data not shown). Increasing the reaction time to 24 h and the enzyme amount to 5 Units, did not improve the conversion of Phe by CrP (data no shown). In contrast, Ant also consisting of three rings was hydroxylated by CrP at two different positions. Phe conversion by AaP was accompanied with the formation of at least eight hydroxylated metabolites (Table 1) and yielded above all *mono*-hydroxylated compounds, whose data corresponded with those reported by Sack et al. (1997a) for 1-OH-Phe, 3-OH-Phe and 4-OH-Phe (1-, 3- and 4-phenanthrol); 9-OH-Phe (9-phenanthrol) available as authentic standard was not detected (see supplementary material Fig. S3). These findings indicate that Phe binds in that way to the enzyme's active site that the carbon atoms of the 1-, 3- and 4-position become accessible to the heme-bound ferryl oxygen. After the formation of these major metabolites, several *di*-hydroxylated metabolites appeared in the reaction solution, which were tentatively identified by their mass spectra (Table 1).

Ant was hydroxylated by AaP to a greater extent and more rapidly than Phe in presence of the radical

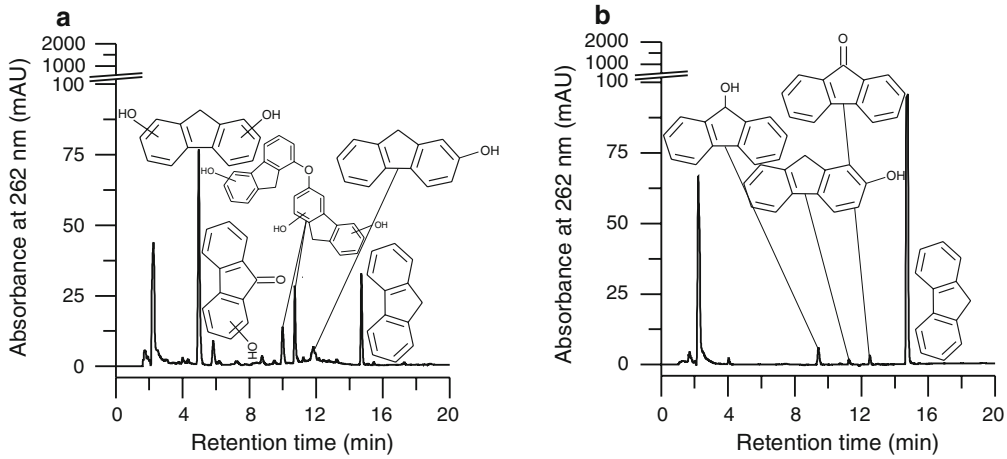


Fig. 3 HPLC elution profiles of metabolites formed during Flu conversion by AaP (**a**) and CrP (**b**). Chromatograms were recorded at 262 nm

scavenger. The formation of several *mono*-hydroxylated and *di*-hydroxylated compounds was inferred from the mass spectra of a number of new peaks in the HPLC elution profiles, but the exact position of the hydroxyl groups could not be ascertained due to the lack of respective standards (Table 1). A putative quinone (1,4-anthracenedione) was also detected in the reaction solution by means of its UV–Vis spectrum and respective literature data, and furthermore, small amounts of a hydroxylated quinone were deducible from its mass spectrum (Table 1). Since authentic standards of 1-anthrone and 1,9-anthraquinone were available, we specifically searched for these compounds but none of them was detectable (see supplementary material Fig. S4).

Conversion of pyrene (Pyr) and perylene (Per)

The insets of Fig. 4 show the UV-spectra of the products obtained after conversion of Pyr by AaP and CrP in the presence of ascorbic acid. It was oxidized into 1-OH-Pyr (1-pyrenol) by both peroxygenases, followed by a second hydroxylation to give 1,6- and 1,8-*di*-OH-Pyr (1,8-dihydroxypyrene) (Table 1; Fig. 1g). 1-OH-Pyr (up to 440 µM in presence of the radical scavenger) was detected as the major metabolite and it was identified by comparing its retention time and UV-spectrum with an authentic standard. The UV-spectra of the *di*-hydroxylated metabolites were assigned to appropriate compounds by comparing their data with the literature (Lambert

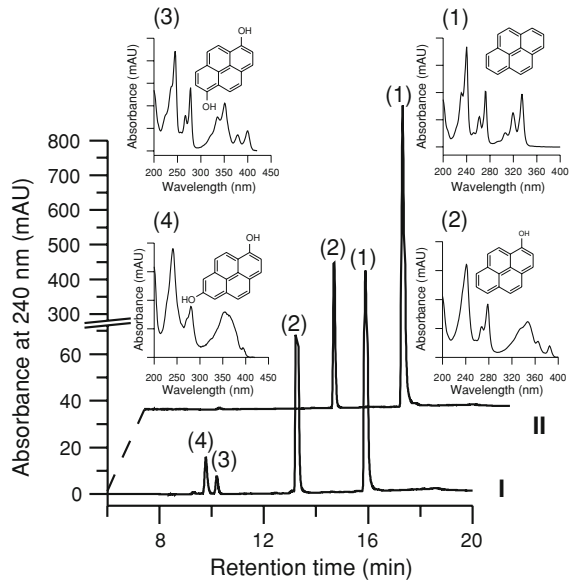


Fig. 4 HPLC elution profiles of metabolites formed in the course of Pyr conversion by AaP (I) and CrP (II) in presence of ascorbic acid. Chromatograms were recorded at 240 nm. (*1*) pyrene, (*2*) 1-hydroxypyrene, (*3*) 1,6-dihydroxypyrene, (*4*) 1,7-dihydroxypyrene

et al. 1994). In the case of CrP, only traces of 1,8-*di*-OH-Pyr were found and again the conversion of the substrate (Pyr) was incomplete and proceeded slowly. Thus, only 13% of Pyr was oxidized by CrP within an 8-hour period compared to 45% converted by AaP (Fig. 4). Typical microbial oxidation products of Pyr

such as 1,6- and 1,8-pyrenequinone were not found like in the experiments with Phe.

The bulky molecule of Per was not oxidized, neither by AaP nor by CrP. Even when the reaction time was increased to 24 h and the amount of enzyme to 10 U ml^{-1}, no conversion of Per was observed (data not shown).

Discussion

The ability of two aromatic peroxygenases (APO) from the basidiomycetous fungi, *A. aegerita* (AaP) and *C. radians* (CrP), was tested to catalyze the oxygenation of a number of polyaromatic compounds. Both enzymes were found to introduce oxygen functionalities into aromatic rings, although the efficiency of AaP was considerably higher than that of CrP. In recent studies on these peroxide-consuming enzymes, we have demonstrated that the origin of oxygen transferred to the substrate molecule is in fact the peroxide (Kluge et al. 2009; Aranda et al. 2009). These results were obtained using $^{18}O_2$-labeled hydrogen peroxide ($H_2^{18}O_2$) as well as naphthalene and dibenzothiophene as substrates. Here we show that an increase in molecule size from two to five rings strongly influences the conversion rate and the extent of hydroxylation. Thus, the conversion rates determined for AaP were negatively correlated to the size of the substrate molecule in the following order: 1-/2-MN > Dbf/Flu > Phe > Ant > Pyr and the five-ring PAH Per was not oxidized at all, i.e. there is a limit regarding the size of the converted molecule. A similar behavior was observed for CrP, which did not attack Per and Phe, and the other substrates as follows: 2-MN > 1-MN > Flu > Dbf > Ant > Pyr.

In comparison to P450 monooxygenases or chloroperoxidase (CPO), the APO proteins showed a remarkable higher specific activity towards most of the studied PAHs with catalytic rates of 170 nmol min^{-1} $nmol^{-1}$ enzyme and 14.3 nmol min^{-1} $nmol^{-1}$ enzyme (AaP) for the conversion of Flu and Phe, respectively (Harford-Cross et al. 2000). In the case of CPO, a higher specific activity compared to APOs is only known for the oxidation of Pyr (according to the data reported by Ayala et al. 2000). It is, however, to consider that CPO does not oxygenate but oxidatively halogenate PAHs.

Major differences between both enzymes (AaP, CrP) were observed regarding the oxidation of 1-MN, 2-MN and Flu. The position of the methyl group had a strong influence on the conversion and determined the site of oxidation. In the case of 2-MN, the methyl group was preferably oxidized by AaP and CrP leading to its stepwise oxidation via naphthalene methanol and naphthaldehyde to naphthoic acid, whereas the methyl group of 1-MN was only the preferred target of CrP while the priority oxidation site of AaP was the aromatic ring. With 1-MN as substrate, the differences between both enzymes were particularly accentuated and the findings indicate that substrate binding in the active site differs between AaP and CrP. In the case of AaP, the substitution at C_1 could sterically hinder the orientation of the molecule in that way that the methyl group cannot come in direct contact with the ferryl oxygen of APO-compound I (Ullrich and Hofrichter 2007). In contrast, the methyl group at C_2 is oriented "in a line" with the aromatic rings so that it may enter the active site under direct contact to the ferryl oxygen. In case of CrP, however, the oxygenation always preferably occurred at the methyl group, maybe due to a lower redox potential of the enzyme compared to AaP or difficulties to correctly place the aromatic ring near the heme. A similar phenomenon was described for PAH oxidation by fungal P450s (Cerniglia et al. 1984).

Differences in the catalytic behavior of AaP and CrP were also observed for the oxidation of Flu. AaP oxygenated only the aromatic rings but did not attack the C_9-carbon. The opposite effect was observed in the case of CrP that preferably oxidized the non-aromatic C_9-carbon of the middle ring leading to the formation of 9-fluorenol and 9-fluorenone as the major products. The formation of 9-fluorenone and traces of 9-fluorenol were also reported for the lipid-mediated Flu oxidation by manganese peroxidase (MnP; Bogan et al. 1996). Flu has a relatively high ionization potential (8.2 eV) so that even high-redox potential peroxidases such as LiP cannot directly oxidize it via one-electron or H-abstraction (Bogan et al. 1996). Only the mediation by lipid oxyradicals makes an H-abstraction (from C_9) possible and leads, via transient 9-fluorenol, to 9-fluorenone. The abundant formation of both metabolites in vivo was demonstrated in a comprehensive screening with 30 fungal species (Garon et al. 2000). Monooxygenases

of the P450 type have been proposed as oxygenating biocatalysts which transfer one O-atom from dioxygen to the C_9 of Flu (Pothuluri et al. 1993). A similar mechanism may also apply to CrP, that was shown to transfer peroxide-oxygen to polycyclic substrates (Aranda et al. 2009). It is interesting to note that—contrary to a recently published report on Flu metabolism by an *Agrocybe* sp. strain (Chupungarsa et al. 2009)—AaP did not produce 9-fluorenol or 9-fluorenone. This behavior resembles own previous observations (Aranda et al. 2009) regarding the conversion of dibenzothiophene (DBT) in vivo and in vitro by both fungi and their peroxygenases: AaP favored the hydroxylation of the aromatic rings of DBT whereas CrP favored the oxidation of the sulphur.

Our results furthermore show that both APOs have a strong peroxidative (i.e. phenol oxidizing) activity that caused the further oxidation of phenolic products in the absence of ascorbic acid. In this way, aromatic peroxygenases resemble CPO that was proposed to have different oxidation sites for peroxidatic substrates, competing with the oxygenation site in the heme channel (Manoj and Hager 2008). Therefore the latter and other authors have characterized CPO as "Janus enzyme" showing properties both of cytochrome P450 monooxygenases and classic heme-peroxidases (Omura 2005; Ullrich and Hofrichter 2007; Brown et al. 2008). The same—and even to a greater extent—applies to APOs, since their oxygenative activities, which include also oxygen transfers to aromatic substrates, are at least as strongly developed as the peroxidative activities (Kluge et al. 2007). Nevertheless there are clear distinctions between both APOs concerning the oxygenation of methyl groups, aliphatic and aromatic carbons (this paper) as well as of hetero atoms such as sulfur (Aranda et al. 2009). In the end, they must be caused by structural differences near the enzymes' active site, which may favor either the transfer of the peroxide oxygen to a non-aromatic position (e.g. S of DBT or C_9 of Flu) or to the adjacent benzene rings (Aranda et al. 2009). All in all, CrP—with its limited ability to oxygenate aromatic rings and the preference for methyl and methylene groups—rather resembles CPO, and can be grouped with respect to its catalytic properties between AaP and CPO. So CPO was found to catalyze oxygen transfers to the methyl group of toluene, to the non-aromatic ring of indene and to the sulfur of thioanisole (Manoj et al. 1999; Park and Clark 2006; Liu and Wang 2007, Manoj and Hager 2008) but it is not capable of oxygenating naphthalene or other PAHs (like Dbf, Flu, Ant, Phe and Per tested in this study, unpublished data). Unlike CPO and CrP, AaP is clearly favoring aromatic rings in its catalysis and in this way, rather resembles P450 monooxygenases, which are the most efficient biocatalysts oxygenating aromatic compounds (Omura 1999). On the other hand, it must be stressed that AaP (and CrP, as well) and P450s do not share any sequence homology whereas the sequence identity of AaP and CPO is—after all—27% (Pecyna et al. 2009). Whether the molecular architecture of APOs and P450s, in particular around the active site, shows functional similarity (e.g. concerning the arrangement of helices, domains or active amino acid residues), will be clarified in ongoing crystal structure analyses (Piontek et al. 2009, unpublished).

Several intracellular fungal enzymes have also been shown to hydroxylate aromatic compounds including PAHs (Cerniglia et al. 1985). Among them there are flavin-dependent monooxygenases (Neujahr and Gaal 1973), besides a number of P450s (Shimada 2006).

The pathways a–g in Fig. 1 summarize the results on PAH conversion by APOs. They differ in parts from those reported in the literature, in particular with respect to the formation of quinones, dihydrodiols and ring cleavage products. The latter is not surprising since APO does neither possess a dioxygenase-like activity to cleave the ring (Vaillancourt et al. 2006) nor an epoxide hydrolase activity that forms dihydrodiols (Yang 1988). One quinone has been detected during Ant oxidation (traces of putative 1,4-anthracenedione) that is in accordance with previous findings on naphthalene (Kluge et al. 2009) but differs from reports on LiP and MnP, where 1,9-anthraquinone was the typical quinoid product of Ant oxidation (Hammel et al. 1991; Kotterman et al. 1994; Eibes et al. 2006) or Laccases (Collins et al. 1996).

A high hydroxylation activity was observed for Dbf, probably because of the presence of heterocyclic oxygen in the molecule, which may provide more reactivity to the aromatic rings and facilitate oxygenation (Altarawneh et al. 2008). Several *mono*- and *di*-hydroxylated metabolites were detected after treatment of Dbf with monoxygenases of the

ascomycetous mold *Paecilomyces lilacinus* (Gesell et al. 2004) or with whole-cells of the zygomycete *Cunninghamella elegans* (Cerniglia et al. 1979). The latter fungus has an enzymatic system that is comparable with the microsomal monooxygenase activities found in rat liver (Zhang et al. 1996). However, products with more than three hydroxyl groups (*tri*-OH-Dbfs) were not found in these studies and to our best knowledge, this is the first proof of the formation of such polyhydroxylated metabolites.

AaP converted Phe into a mixture of 1-, 3- and 4-OH-Phe (phenanthrols), which is again similar to the activities reported for unspecific monooxygenases of *C. elegans* (Cerniglia and Yang 1984) and for engineered P450 enzymes (Harford-Cross et al. 2000). The inability of CrP (and of course of CPO) to oxygenate Phe must be, again, a question of the molecular architecture of the enzyme's active site. In general, bacteria oxidize Phe preferably by dioxygenases attacking the molecule at the bay-region (i.e. at the C_3 and C_4 or C_5 and C_6 positions), to form Phe *cis*-dihydrodiols (Cerniglia and Yang 1984) but there are also some bacterial species which metabolize Phe at the K-region (i.e. at C_9 and C_{10}), as mammalian enzymes do (Miura et al. 1968). Fungal conversion of Phe was reported to be initiated by P450 monooxygenases leading to mixtures 9- and 10-OH-Phe or Phe trans-1,2-, 3,4-, 9,10-dihydrodiols (Sutherland et al. 1991; Hammel et al. 1992). LiP of *P. chrysosporium* was not able to oxidize Phe but MnP did it in the presence of suitable co-oxidants (glutathione, Tween 80) and formed quinoid metabolites (Hammel et al. 1992; Sack et al. 1997b; Steffen et al. 2002). The lack of hydroxylation at the positions 9 and 10 of Phe by AaP leads us postulate that the correct positioning of the molecule in the heme channel may occur via involvement of the carbon atoms at the positions 1, 3 and 4. The results of Pyr conversion by AaP (and to some extent also by CrP) confirmed the findings made for Phe. Again, *di*-hydroxylated oxidation products were detected but in contrast to Phe oxidation, a variation in the position of the first hydroxyl group was not observed (only 1-OH-Pyr but no 2-OH-Pyr was detectable). This is in accordance with earlier findings using fungal cultures and human liver cells, where 1-OH-Pyr was also the major initial product (Sack et al. 1997a; Sohl et al. 2008). LiP and to some extent also MnP were found to directly attack Pyr leading via a number of postulated radical intermediates to the formation of 1,6-Pyr quinone (Cerniglia et al. 1986; Hammel 1995).

The inability of both APOs to oxidize Per clearly points to limitations of the enzymes concerning the size of the molecule to be oxygenated. This is not surprising since oxygenation requires the direct contact of the substrate with the ferryl oxygen of compound I, which is simply impossible from a certain molecule size upwards (Hofrichter and Ullrich 2006; Isin and Guengerich 2008). On the other hand, this finding also suggests that the active site must be somehow buried in the protein and that an oxygen transfer by a heme exposed at the biocatalyst's surface can be excluded (unlike oxygenations catalyzed by the small heme-containing peptide of microperoxidase; Marques 2007).

All in all, our results indicate a high efficiency of secreted fungal APOs in PAH conversion, which could be of general environmental relevance due to the habitats, in which these fungi occur and due to the metabolites formed: hydroxylated PAHs susceptible for further degradation. Since only two enzymes of this type have been compared so far, more peroxygenases and among them maybe even more efficient biocatalysts are to expect. In that respect the huge biodiversity of the fungal kingdom has to be specifically screened with molecular tools for APO-like sequences (Pecyna et al. 2009).

Acknowledgments Funding of this research is gratefully acknowledged to the Spanish Science and Technology Foundation (FECYT) and MICINN (grant to E. Aranda), the European Union (integrated project BIORENEW), the "Deutsches Bundesministerium für Bildung, Wissenschaft und Forschung" (BMBF; project 0313433D) and the "Deutsche Bundesstiftung Umwelt" (DBU; project 13225-32). Special thanks go to M. Kluge and M. Kinne for their analytical advice and C. Liers, M. Poraj-Kobielska and M. Pecyna for their interesting discussions. We thank U. Schneider and M. Brant for their excellent technical assistance.

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