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Molecular characterization of aromatic peroxygenase from *Agrocybe aegerita*

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Abstract Recently, a novel group of fungal peroxidases, known as the aromatic peroxygenases (APO), has been discovered. Members of these extracellular biocatalysts produced by agaric basidiomycetes such as *Agrocybe aegerita* or *Coprinellus radians* catalyze reactions—for example, the peroxygenation of naphthalene, toluene, dibenzothiophene, or pyridine—which are actually attributed to cytochrome P450 monooxygenases. Here, for the first time, genetic information is presented on this new group of peroxide-consuming enzymes. The gene of *A. aegerita* peroxygenase (*apo*1) was identified on the level of messenger RNA and genomic DNA. The gene sequence was affirmed by peptide sequences obtained through an Edman degradation and de novo peptide sequencing of the purified enzyme. Quantitative real-time reverse transcriptase polymerase chain reaction demonstrated that the course of enzyme activity correlated well with that of mRNA signals for *apo*1 in *A. aegerita*. The full-length sequences of *A. aegerita* peroxygenase as well as a partial sequence of *C. radians* peroxygenase confirmed the enzymes' affiliation to the heme-thiolate proteins. The sequences revealed no homology to classic peroxidases, cytochrome P450 enzymes, and only little homology (<30%) to fungal chloroperoxidase produced by the ascomycete *Caldariomyces fumago* (and this only in the N-terminal part of the protein comprising the heme-binding region and part of the distal heme pocket). This fact reinforces the novelty of APO proteins. On the other hand, homology retrievals in genetic databases resulted in the identification of various APO homologous genes and transcripts, particularly among the agaric fungi, indicating APO's widespread occurrence in the fungal kingdom.

Keywords Peroxygenase · Chloroperoxidase · Cytochrome P450 · Heme-thiolate · Oxygenation · *Coprinellus*

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Introduction

Few years ago, an exceptional peroxide-consuming enzyme—*Agrocybe aegerita* peroxidase—was discovered in the agaric Black poplar mushroom (Ullrich et al. 2004). The purified enzyme was found to be a heme-thiolate protein as indicated by its characteristic spectroscopic properties (Ullrich and Hofrichter 2005). Catalytic studies revealed that *A. aegerita* peroxidase is able to perform reactions

formerly only assigned to intracellular cytochrome P450 monooxygenases (P450s; catalytical potential is reviewed in Bernhardt (2006) and references therein) or to extracellular chloroperoxidase (CPO; catalytical properties are discussed in Ullrich and Hofrichter (2007) and references therein).

Among other reactions, *A. aegerita* peroxidase catalyzes the regioselective epoxidation/hydroxylation of naphthalene, the sulfoxidation of dibenzothiophene and thioanisole, the *N*-oxidation of pyridine, the *O*-dealkylation of alkylaryl ethers, the oxidation of aryl alcohols and aldehydes as well as the bromination of phenol (Aranda et al. 2008; Kinne et al. 2008; Kluge et al. 2007; Ullrich et al. 2008; and unpublished results). These reactions including oxygen transfers are catalyzed in vitro solely by the purified enzyme in the presence of hydrogen peroxide acting as electron accepting cooxidant and oxygen source (Kluge et al. 2009). This catalytic behavior accounted for the renaming of the enzyme into *A. aegerita* peroxygenase (abbreviated as AaP; Ullrich et al. 2008) and later, due to the identification of similar biocatalysts in other fungi, we proposed to designate these enzymes as aromatic peroxygenases (APO) by following the term "aromatic monooxygenases" used for certain P450 enzymes (Zhou et al. 1999).

The haloperoxidase activity of APO resembles that of CPO from the ascomycete *Caldariomyces fumago* but the oxygen transfer reactions which include various aromatic oxygenations are unique among peroxidases. After discovery and purification of a second peroxygenase capable of aromatic peroxygenation from the coprophilous fungus *Coprinellus radians* (*C. radians peroxygenase*, abbreviated as CrP; Anh et al. 2007) and the detection of relevant enzyme activities in *Coprinopsis verticillata*, *Marasmius* sp., and other *Agrocybe* spp. (unpublished results), it is assumed that a novel group of fungal oxidoreductases (probably the second peroxidase sub-subclass; EC 1.11.2.x) has been found. Though AaP and CrP were purified and characterized regarding their physical and catalytic properties, the encoding genes have not been identified so far. Thus, here we present conclusive data from molecular studies on fungal peroxygenases including, for the first time, a full-length nucleotide sequence of an aromatic peroxygenase. Furthermore, the characteristic features of *A. aegerita* cultivation in soybean medium stimulating AaP production were studied along with gene expression by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

Materials and methods

Culture conditions, enzyme activity measurement, and protein purification Cultivation of *A. aegerita* strain TM-A1 (deposited at the German collection of microorganisms and cell cultures—DSMZ, collection number DSM 22459) and *C. radians* strain DSMZ 888 was performed in 3% (*w*/*v*) soybean flour medium ("Hensel Voll-Soja", Schoenenberger GmbH, Magstadt, Germany) in agitated Erlenmeyer flasks at 24°C. For comparison, the *A. aegerita* strain CBS 127.88 that produces low amounts of APO was used. APO activity was measured with veratryl alcohol, and the proteins were purified by fast protein liquid chromatography as described previously (Ullrich et al. 2004; Ullrich and Hofrichter 2005; Anh et al. 2007).

Determination of amino acid sequences Sequences of AaP and CrP on the protein level are based on earlier studies (Anh et al. 2007; Ullrich et al. 2004) and were repeatedly determined by de novo sequencing of the isoform-pure protein fraction II of AaP prepared by Mono P separation (R. Ullrich, unpublished data) and an equally pure fraction of CrP using nanoliquid chromatography/mass spectrometry–mass spectrometry and matrix assisted laser desorption/ionization-time of flight (Protagen Co., Dortmund, Germany). Experimentally determined masses of tryptic peptides were compared with theoretical peptide masses calculated using the program PeptideMass (Wilkins et al. 1997) and the cDNA sequence of AaP translated into amino acids (peptide mass fingerprinting).

Enzymatic deglycosylation of AaP Purified isoform AaP II (12.5 μg) was denaturated with sodium dodecyl sulfate (SDS) and deglycosylated for 3 h with the Enzymatic Protein Deglycosylation Kit from Sigma (Saint Louis, MO, USA). The latter one contained PNGase F, *O*-glycosidase, two α-2(3,6,8,9) neuramidases as well as β-1,4-galactosidase and β-*N*-acetylglucosaminidase and was used according to the instructions of the provider. Molecular mass of the deglycosylated protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE) using a 12% gel.

Isolation of nucleic acids and cDNA synthesis Mycelium of agitated cultures of *C. radians* (strain DSMZ 888, cultivation day 12) and *A. aegerita* (strain TM A1, cultivation day 16) was separated from the culture liquid (200 mL) by filtration. After lyophilization of the mycelium using an Alpha 2–4 freeze dryer (Christ, Osterode, Germany), genomic DNA was isolated using a protocol previously described by Nikolcheva and Bärlocher (2002). Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA, which was stored at −80°C.

For cDNA synthesis, total RNA (1 μg) was primed by using a poly(dT)-anchor primer. Afterward, the total mRNA was reversely transcribed into cDNA with the anchor sequence added to the 3′ end using a "RevertAid H Minus First Strand cDNA Synthesis Kit" (Fermentas, St. Leon-Rot, Germany). Furthermore, by adding 1 μl primer TS-

short (10 μM) to the reaction mix, an anchor sequence was added to the 5′ end of the cDNA using a protocol according to Matz et al. (1999).

PCR conditions For conventional PCR amplifications, a "MasterCycler EP Gradient S" gradient cycler (Eppendorf, Hamburg, Germany) was applied. All primers were obtained from Eurofins-MWG-Operon (Ebersberg, Germany). Primers used in this study are listed in Table 1.

The PCR reaction mixtures (25 μL) contained 10 μL PCR Master Mix ("HotMaster Mix", 2.5-fold concentrated; 5Prime, Hamburg, Germany), 1 μL of each primer from 10 μM stock solution in case of specific primers and from 100 μM stock solution for degenerated primers, 1 μL of cDNA, and 12 μL PCR grade water. The PCR was started with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 50.4°C (in case of degenerated primers) or temperatures according to the "4+2 rule" (in case of specific primers; Rychlik and Rhoads 1989) for 45 s, and elongation at 72°C for 1.5 min. Final elongation took 10 min at 72°C. Resulting PCR products were purified ("SureClean", Bioline, Luckenwalde, Germany) and cloned in *Escherichia coli*.

Cloning, sequencing, and sequence analysis Plasmids derived from dU/A-cloning of PCR fragments with the "pSTBlue-1 AccepTor Vector Kit" (Merck (Novagen), Darmstadt, Germany) were verified by colony PCR (Woodman 2008) and several independent clones were used for sequencing. Sequencing was performed on ALFexpressII equipment in combination with an AutoRead Sequencing Kit (both GE Healthcare, Munich, Germany). Software BioEdit 7.0.9 was used for sequence analyses (Hall 1999). The program iPSORT (Bannai et al. 2002) was used to calculate the Kyte–Doolittle hydrophobicity index. Possible glycosylation sites were predicted using the programs NetNGlyc 1.0 (available via http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 (Julenius et al. 2005). Further parameters of the deduced protein such as the molecular weight and isoelectric point (pI) were calculated using the ProtParam tool (Gasteiger et al. 2005). Protein fold recognition was performed on the phyre web server (Bennett-Lovsey et al. 2008; available via http://www.sbg.bio.ic.ac.uk/phyre/).

Sequencing strategy for the A. aegerita apo1 gene By using the peptide sequences of two internal peptide fragments of AaP (Anh et al. 2007), the degenerated primers AaP2-Rev and AaP4-Rev were designed. These primers were used on the cDNA level to amplify fragments of a peroxygenase gene from *A. aegerita* (strain TM-A1). A 5′ rapid amplification of cDNA end (RACE) experiment (Matz et al. 1999) was performed with the specific primer SO-Mix (containing 90% heel-specific primer and 10% heel-carrier primer, 10 μM) and the degenerated primer AaP4-Rev. The 1:100 diluted PCR product was then used in a nested PCR with the SO-Mix and degenerated primer AaP2-Rev. The resulting band with a size of approximately 350 bp was excised from the gel, purified, and cloned. Several independent clones were fully sequenced. Based on these data, the specific primer 1AaP-For1 was designed.

For completion of the sequence at the cDNA level, a 3′ RACE experiment (Frohman et al. 1988) was performed:

Table 1 Oligonucleotides used as primers

Primer name	Sequence (5′ → 3′)
Poly(dT)-anchor primer	TAGCTCGATGCTTGCACGCTTTTTTTTTTTTTTT
AP primer	TAGCTCGATGCTTGCACGC
TS-short primer	AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
Heel-carrier primer	GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Heel-specific primer	GTAATACGACTCACTATAGGGC
Aap2-Rev	GCIARNGTRTTIARICCNGG
Aap4-Rev	AARTCIGGRTTNGTNGC
Cop1-For	CCICCNCCIGARTAYGT
Cop6-Rev	CCARAARTCRTCNGGCAT
1Aap-For1	TTCTACATGAAATATTTTCC
1Aap-Rev2	AAGCAGGTTGTTGGACCG
Scaap	*FAM*-TAGTTATACAGGCTCATCCTATGCAG-*TAMRA*
fpcaap	CACCCAGCCCGAGAAGTGGCACA
rpcaap	TGTAGCTGTTGATCTTGCCGACATTTC
S5.8	*FAM*-TAAGTAATGTGAGTTGCAGAATTCAGTGAA-*TAMRA*
fp5.8	CGATGAAGAACGCAGCGAAA
rp5.8	GGAATACCAAGGAGCGCAAGG

Sequences are written according to International Union of Pure and Applied Chemistry nucleotide code, and *FAM* and *TAMRA* denote dyes labeled to oligonucleotides (hydrolysis probes)

rG ribonucleotide guanosine, *I* inosine wobble base, *FAM* carboxyfluorescein, *TAMRA* carboxytetramethylrhodamine

PCR with the primer combination 1AaP-For1 and 3′ anchor primer AP resulted in an about 1,300-bp long fragment. This fragment was cloned; two clones were fully sequenced and revealed the whole cDNA sequence of a putative AaP gene, designated as *A. aegerita apo*1. This sequence was identified as a homolog to CPO sequence by a Basic Local Alignment Search Tool (BLAST) search. After completion of cDNA sequence, specific primers 1AaP-For1 and 1AaP-Rev2 were used in PCR to amplify complete CDS and 3' untranslated region of the AaP gene from genomic DNA (about 1,500 bp). The PCR product was purified, cloned, and finally, two independent clones were fully sequenced.

Sequencing strategy for the C. radians apo1 gene Based on the knowledge of the peptide sequence of the N terminus (Anh et al. 2007) and one internal peptide fragment (this study) of the CrP protein, degenerated primers Cop1-For and Cop6-Rev were designed and used on cDNA level to partially amplify a fragment of the peroxygenase gene from *C. radians*. The PCR product, which derived from the application of both degenerated primers (size of approximately 700 bp), was purified from agarose gel, cloned, and sequenced. The obtained sequence was recognized as a putative homologue to the CPO sequence by a BLAST search.

Expression analysis of the A. aegerita apo1 gene by quantitative real-time reverse transcription PCR For qRT-PCR experiments, *A. aegerita* strain TM-A1 and *A. aegerita* strain CBS 127.88 were cultivated in soybean flour medium (3% *w*/*v*) in agitated flasks (200 mL suspension, 100 rpm) in triplicate for 42 days at 24°C. Samples (1 mL) were collected at intervals of 3 to 5 days. After centrifugation, the pH as well as peroxygenase activity was determined in the supernatant; biomass was obtained from the precipitated pellet. This fresh biomass (200 mg each sample) was snap freezed in liquid nitrogen and stored at −80°C. Total RNA was extracted by "TRIzol Plus RNA Purification Kit" (Invitrogen) and was spectrophotometrically quantified by determining the optical density at 260 nm. Integrity of RNA was checked by agarose gel electrophoresis. Afterward, RNA was treated with RNase-free DNaseI (Fermentas). For reverse transcription of the RNA samples, "RevertAid H Minus First Strand cDNA Synthesis Kit" (Fermentas) was used with random hexamer primers according to the manufacturer's instructions.

For qRT-PCR amplifications, a "Rotor-Gene 3000" cycler (Corbett Research, Sydney, Australia) with a 36-well rotor was applied. The PCR reaction mixtures (25 μL) in 0.2 mL tubes contained 10 μL PCR Master Mix ("RealMasterMix Probe", 2.5-fold concentrated; 5Prime, Hamburg, Germany), 0.5 μL of each primer from 15 μM stock solution, 0.5 μL of dual-labeled probe from 10 μM stock solution, 2 μL of cDNA or 5 μL of plasmid DNA, 5 μL $MgSO_4$ (25 mM), and PCR grade water. The PCR program comprised of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation (at 94°C for 15 s) and annealing as well as elongation (at 60°C for 30 s). Carboxyfluorescein (FAM) fluorescence at 510 nm (gain factor 5) was measured at the end of each cycle after excitation at 470 nm.

For both the *A. aegerita* TM-A1 *apo*1 mRNA sequence and the *A. aegerita* TM-A1 5.8 S-rDNA gene (unpublished data), specific primers and dual dye-labeled probes (5′-FAM, 3′-carboxytetramethylrhodamine) were designed (Table 1), purchased from Eurofins MWG Operon and applied on plasmid DNA preparations in several dilutions containing these genes for the determination of standard curves in quantitative real-time PCR. Optimal threshold was selected in the exponential phase of amplification by the least-square fit method. Application of primers fpcaap and rpcaap in cDNA as well as genomic DNA resulted in an *apo*1-specific amplicon of 93 bp in length, which was detected by hydrolysis probe Scaap. The application of the primer pair fp5.8 and rp5.8 resulted in a 5.8S-specific amplicon of 96 bp in length, which was detected by hydrolysis probe S5.8.

Samples from cultivation days 7, 11, 14, 18, 23, 25, 28, 30, 35, and 42 were measured in quadruplicates. No-template controls as well as standards (five dilutions in common logarithm steps of plasmid containing either gene of interest or reference gene) were performed in duplicate during each amplification run. Software Rotor-Gene 6.1 build 93 (Corbett Research) was used for analysis and raw fluorescence data were processed by dynamic tube background subtraction and noise slope correction. Threshold and linear equation of external standard curve were imported; linear equation was adjusted to the standards in every single run (slope of linear equation of standard curve was fixed to external standard, only *y*-intercept was adjusted). Target copy numbers were calculated as a function of threshold cycle based on the linear equation of the obtained standard curve. Finally, in order to evaluate the relative expression of *A. aegerita apo*1 gene, the ratio of average copy numbers of quadruplicates of the tested genes (*apo*1/5.8S) was calculated and calibrated to day 11. The relative error of the expression ratio was estimated as the square root of the sum of the squares of the two relative errors (coefficient of variation) of the calculated mean copy number.

Phylogenetical analyses By performing BLASTn, BLASTx, and tBLASTx searches in December 2008 (BLAST; Altschul et al. 1997) with CPO, AaP, and CrP nucleotide sequences, homologous nucleotide sequences were identified (Table 3). Translated sections of messenger RNA and expressed sequence tag (EST) sequences comprising an

approximately 100 amino acid long sequence from the heme-binding region to the second conserved region (EGD motif in the distal heme environment) were aligned by program ClustalW 2.0 with standard parameters (Larkin et al. 2007), visually revised, and modified if necessary using BioEdit. Following species were excluded from multiple alignment due to the incompleteness in this area: *Pleurotus ostreatus*, *Amanita muscaria*, *Hebeloma cylindrosporum*, *Uromyces appendiculatus*, and *Hyperamoeba dachnaya*. The phylogeny was assessed with randomized input order using maximum likelihood (with global rearrangements) and maximum parsimony analysis as implemented in Phylip package (Felsenstein 1989, 2005), being based on mentioned protein sequence alignment of three true and 12 putative fungal peroxygenase sequences and 110 amino acid positions. Branch support was estimated using 1,000 bootstrap replicates.

Accession numbers Sequences of *A. aegerita* and *C. radians apo* genes were deposited in European Molecular Biology Laboratory nucleotide database under following accession numbers: FM872457 (*A. aegerita apo*1 gene), FM872458 (*A. aegerita apo*1 mRNA), and FM872459 (*C. radians apo*1 partial mRNA).

Results

Gene sequence of an aromatic peroxygenase from A. aegerita A gene designated as *A. aegerita apo*1 was found to be transcribed into mRNA in liquid cultures of *A. aegerita* showing peroxygenase activity, and it was completely sequenced at the cDNA (1,279 bp) and genomic DNA (1,489 bp) level (Fig. 1). The deduced open reading frame of 1,374 nt encodes for 371 amino acids and contains five introns. The introns in the range from 49 to 59 nt follow the GT–AG rule (Shapiro and Senapathy 1987). By comparing the theoretical peptide masses after tryptic digestion of the putative *apo*1 gene product and the real masses of tryptic peptides derived from an isoform-pure AaP protein preparation, *apo*1 was confirmed to be the gene encoding for AaP (Table 2). Eight peptides, perfectly matching and equally distributed across the whole sequence of the *apo*1 gene product, were identified, i.e., a 35% sequence coverage was achieved. Furthermore, the N-terminal sequence of the mature AaP protein derived from an earlier Edman analysis (Ullrich et al. 2004) fits quite well with the found gene sequence *apo*1 (80% identity). In addition, one de novo peptide confirms the first amino acids of the mature protein AaP (Table 2). These data furthermore indicate that an initial precursor of 371 amino acids will be posttranslationally cleaved to form the 328 amino acid long mature protein that starts with an unusual glutamic acid residue (Fig. 1).

Predicted features of AaP The predicted molecular mass and pI of the mature AaP protein without glycosylation are 35.9 kDa and 5.52, respectively. These data correspond well with those experimentally determined for the purified AaP protein: 37 kDa (after enzymatic deglycosylation; Fig. 2) and pI values between 4.9 and 5.7 (the major isoform has a pI of 5.3; Ullrich et al. 2004). Six potential *N*-glycosylation sites were identified, among which that at position 161 was predicted to be the most likely one. Furthermore, the possible *N*-glycosylation site at the amino acid position 11 is located at a homologous region of CPO (at the amino acid position 12), which is high-mannose *N*-glycosylated (see also Fig. 4; Sundaramoorthy et al. 1995). No indication for the presence of an *O*-glycosylation site was found.

According to the gene sequence, the mature protein AaP contains three cysteine residues. One cysteine (Cys_{36}) in the putative heme-binding region may act as the fifth ligand (axial thiolate ligand) of the central iron (Fe^{3+}) in the porphyrin ring; the two other cysteines were found in the C-terminal region. The Kyte–Doolittle hydrophobicity index shows more hydrophobic residues around these C-terminal cysteines and more hydrophilic residues between them. Since disulfide bonds are mostly found in secretory proteins or exoplasmatic domains of membrane proteins, it is to assume that both cysteines form a Cys_{278}–Cys_{319} disulfide bond exposing the region between them to the hydrophilic outer surface of the protein.

Expression studies of aromatic peroxygenase from A. aegerita After demonstrating that the *apo*1 gene of *A. aegerita* corresponds to the protein previously characterized as AaP, the induction and the expression pattern of *apo*1 were studied by quantitative real-time PCR accompanied by enzymatic measurements. Though the actual inducer of peroxygenase in *A. aegerita* TM-A1 is not yet known, enzyme production can be specifically triggered in media containing soybean flour. Figure 3a shows representative results of a cultivation in soybean medium of three parallel experiments with TM-A1. Within the first 14 days of cultivation, the gene *apo*1 (relative to 5.8S rDNA expression) was transcribed at a low basal level, which corresponds to low enzyme activities near the detection limit (5–20 U/L). Between days 14 and 18, induction took place resulting in gene activation and the drastic rising of *apo*1 expression between days 18 and 23. Activation was followed by a respective increase in enzyme activity in the medium. After day 28, the expression of *apo*1 dropped noticeably and declined to the basal level on day 42. After decrease of the mRNA signal, the enzyme was still present

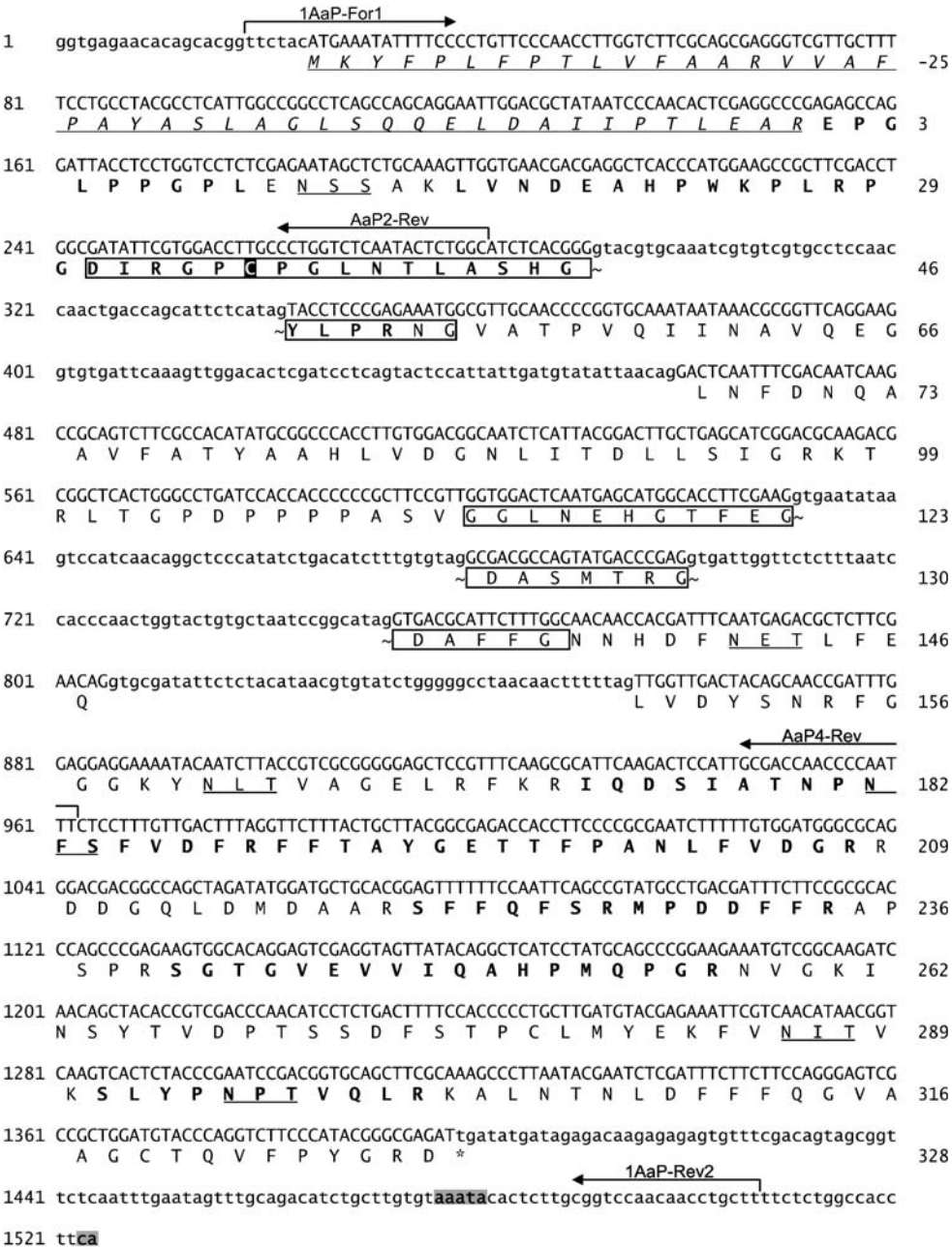

Fig. 1 Nucleotide sequence and deduced amino acid sequence of the *A. aegerita* peroxygenase gene *apo*1. The signal peptide sequence is *underlined* and written *in italics*. The heme-binding region with the cysteine residue serving as axial thiolate ligand as well as the second conserved domain (putative part of the distal heme pocket) are highlighted by *boxes* (interruptions by introns are marked with the *tilde sign*). Possible *N*-glycosylation sites are *underlined*. *Lower case letters* represent intron sequences and complete untranslated regions. The polyadenylation signal consisting of the positioning element and the associated actual site of polyadenylation are shown in the downstream region in *gray shading* (Zhao et al. 1999). The amino acids of the eight perfectly matching tryptic peptides and one partly matching peptide identified by de novo peptide sequencing and peptide mass fingerprinting of the *A. aegerita* peroxygenase protein are marked in *bold letters*. Position, length, and orientation of used primers are marked by *arrows*. The nucleotide sequence beyond the primer binding sites of 1AaP-For1 and 1AaP-Rev2 was only confirmed by cDNA sequencing. The nucleotide sequence is numbered on the *left*, the amino acid sequence on the right side (numbering starts with the first amino acid of the mature protein, i.e., at the first amino acid after the signal peptide)

in the culture liquid due to the high stability of the APO protein. In summary, the experiment clearly demonstrates that enzyme activity almost perfectly follows the mRNA signal and always appeared later than the mRNA signal.

The time course of the absolute copy number for the 5.8S rRNA is shown in Fig. 3b and proves the applicability of 5.8S rRNA for normalization in qRT-PCR. Between days 11 and 35, the absolute copy number of this ribosomal RNA component changed only slightly. Thus, changes in the expression level of *apo*1 between days 11 and 35 were mainly caused by changes in the number of *apo*1-mRNA copies.

As a control, *A. aegerita* strain CBS 127.88 (that secretes 50–100 times less peroxygenase than TM-A1; Ullrich et al. 2004) was studied in parallel to strain TM-A1. Throughout the cultivation, it showed only low peroxygenase activity (comparable to those of *A. aegerita* TM-A1 during the first days of culturing) and the expression level of *apo*1 in CBS 127.88 changed only marginally. The highest expression level was observed for day 18 with a fivefold increase (data not shown); however, compared to the 27-fold increase in expression in TM-A1 at the same day, this change is negligible.

Partial gene sequence of an aromatic peroxygenase from C. radians One gene fragment (denoted as *C. radians apo*1, 687 bp) was identified at cDNA level by PCR using de-

Table 2 Assigned peptide masses, de novo peptide sequences, and N-terminal protein sequence (from Edman degradation) for the *apo*1 gene product of *A. aegerita*

	Experimental mass of tryptic peptides from isoforms of purified *A. aegerita* peroxygenase enzyme (this study)			Predicted mass	Length in amino acids	Position of peptide	Sequence
	pI 5.7 (m/z)	pI 5.27 (m/z)	pI 6.1 (m/z)				
1	–	918.4467163	918.4440918	918.4468	7	221–227	SFFQFSR
2	–	927.4025879	–	927.4029	7	228–234	MPDDFFR
3	1,287.698608	1,287.695435	1,287.680054	1,287.7055	11	291–301	SLYPNPTVHVR
4a	1,862.946899	1,862.942139	1,862.931641	1,862.9541	18	240–257	SGTGVEVVIQAHPMQPGR
4b	1,878.950562	1,878.961426	1,878.931763	1,878.9490	18	240–257	SGTGVEVVIQAHPMQPGR (Met oxidized)
5	1,970.956177	1,970.952271	1,970.947144	1,970.9606	17	173–189	IQDSIATNPNFSFVDFR
6	–	2,113.124268	–	2,113.1301	18	16–33	LVNDEAHPWKPLRPGDIR
7	2,153.033936	2,153.031982	2,153.033936	2,153.0338	19	190–208	FFTAYGETTFPANLFVDGR
8	De novo peptide sequence obtained from Anh et al. 2007				17	34–50	GPCPGLNTLASHGYLPR
9a	De novo peptide sequence obtained from one tryptic peptide of purified *A. aegerita* peroxygenase enzyme mixture (pI 4.9–5.7) (this study)		m/z 1,694.8588	Probability 67.85	Length 16	Position of peptide	Sequence EPGLPPGPLQDMPFGK
9b	Peptide sequence obtained by Edman degradation from earlier study (Ullrich et al. 2004)				14		EPGKPPGPPXESSA
	Peptide sequence of N terminus derived from *apo*1 gene sequence of *A. aegerita*				17	1–17	EPGLPPGPLENSSAK

generated primers, which were based on known amino acid sequences of the N terminus (Anh et al. 2007) and one tryptic peptide from CrP (DHKMPDDFWR; this study). The N-terminal fragment of this putative CrP encoding gene shows 65% identity and 75% similarity, respectively, to the *apo*1 sequence of *A. aegerita*. Three possible *N*-glycosylation sites were found in this sequence confirming the heavily glycosylated nature of the protein (37% glycosylation; Anh et al. 2007).

Discussion

Two new members of the heme-thiolate protein family have recently been described for the agaric mushrooms *A. aegerita* and *C. radians*, and according to their catalytic properties, they have been characterized as APO (Aranda et al. 2008; Kluge et al. 2009; Ullrich et al. 2008; Hofrichter and Ullrich 2006). They combine unique capabilities of P450s (oxygen transfer) and "classic" properties of peroxidases (phenol oxidation, halide oxidation). In the present study, we were able to obtain full-length mRNA and DNA sequences of APO from *A. aegerita* and a partial mRNA sequence of APO from *C. radians*.

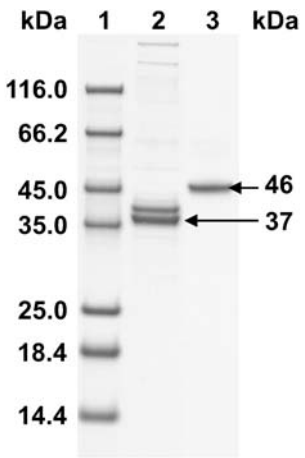

Fig. 2 SDS-PAGE of enzymatically deglycosylated *A. aegerita* peroxygenase (isoform AaP II). *Lane 1* protein standard (protein molecular mass marker, Fermentas, St. Leon-Rot, Germany), *lane 2* enzymatically deglycosylated AaP II, *lane 3* glycosylated AaP II (SDS-PAGE 12%; staining with Colloidal Blue from Invitrogen, Carlsbad, CA, USA). The main band of the deglycosylated protein has a molecular mass of 37 kDa; the other bands represent incompletely deglycosylated AaP; the weak bands in the high-molecular mass region (50–150 kDa) are proteins from the deglycosylation kit

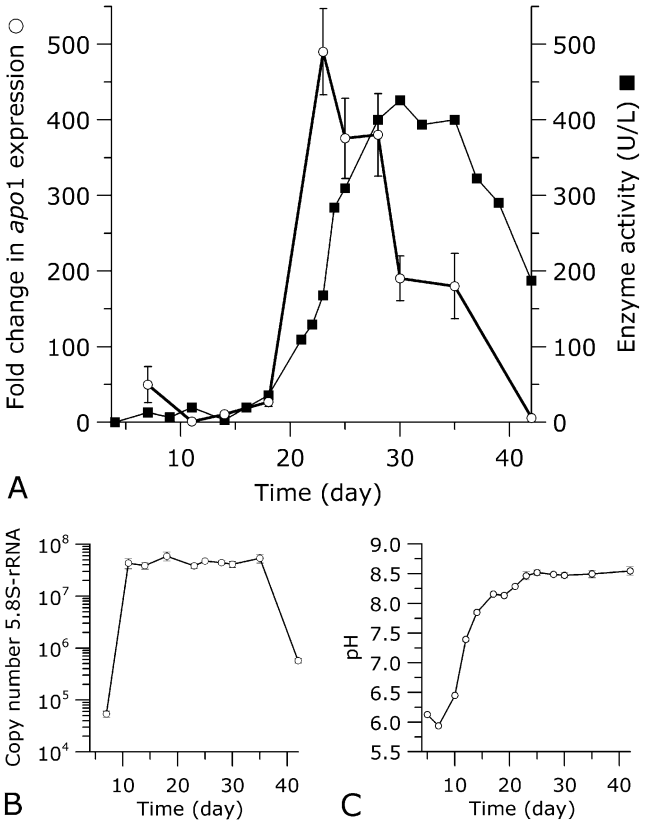

Fig. 3 **a** Time course of relative expression level of *apo*1 gene and peroxygenase production in *A. aegerita* strain TM-A1 while growing in a soybean-based medium. Both assays were performed in independent triplicates; only one representative result is shown. Gene expression levels of *apo*1 (*empty circles*) were detected by qRT-PCR with gene-specific probes; obtained copy number for *apo*1-mRNA was normalized by copy number of 5.8S rRNA from the same sample; each calculated ratio was then calibrated to the ratio of day 11. Each *error bar* shows propagated standard deviation derived from quadruplicate measurements from one sample. Enzymatic activity (*filled squares*) was measured by determining the peroxidation of veratryl alcohol into veratrylaldehyde at pH 7 (Ullrich et al. 2004). Data points represent mean values for three repeated measurements (coefficient of variation <5%). **b** 5.8S-rRNA copy number as detected by qRT-PCR (nonnormalized same samples used as in **a**). Standard deviation is given as *error bars*. **c** Time course of pH in the culture supernatant (same samples used as in **a**)

Heme-thiolate proteins bear a protoporphyrin IX (heme) as prosthetic group in the active site that is coordinated by the thiolate anion of a cysteine residue (fifth or axial ligand). They constitute one of the two major heme-protein groups found in living organisms; the other one is the heme-imidazole group (bearing a histidine residue instead) that includes hemoglobin and most heme-peroxidases (e.g., horseradish peroxidase, ligninolytic peroxidases; Ortiz de Montellano 1992; Kincaid et al. 1979). The intracellular P450s are ubiquitous in all kingdoms of life and were the first heme-proteins found to contain a thiolate residue as the axial ligand. A few other heme-thiolate proteins including mammalian nitric oxide synthase and fungal chloroperoxidase were later found (Omura 2005). All these heme-thiolate enzymes as well as the APOs described here can catalyze the transfer of oxygen functionalities to various substrates, even if to different extents (Ullrich and Hofrichter 2007).

BLAST searches for protein sequences Based on the nucleotide sequences of aromatic peroxygenases (AaP and CrP), BLASTp searches produced no matches with any heme-imidazole peroxidase (i.e., ligninolytic or phenol oxidizing peroxidases) or P450s. On the other hand, the sequences of AaP and CrP matched to some extent (maximum 27% identity) with that of CPO from the ascomycete *C. fumago (Leptoxyphium fumago)*. Protein domain and motif scan performed on the website of the Swiss Institute of Bioinformatics (Hulo et al. 2008) confirmed this exclusive match with CPO and corresponds to the earlier observation of N-terminal similarity of AaP and CPO (35% identity; Ullrich et al. 2004). However, this similarity only concerns the N-terminal moiety of CPO comprising the proximal heme-binding region and part of the distal heme pocket (see also Fig. 4). Comparison of these sequences revealed that the heme-binding regions of *A. aegerita* and *C. radians* peroxygenases contain a conserved cysteine residue (in both proteins Cys_{36}) that serves in CPO as the fifth heme ligand (Cys_{29}). This finding confirms on the molecular level the enzymes' affiliation to the heme-thiolate proteins, which had already been deduced from spectral studies (Ullrich and Hofrichter 2005). However, it must be stressed that the AaP sequence differs completely from that of CPO in the C-terminal part, which may explain the considerable differences of both enzymes concerning their catalytical properties (e.g., oxygenation of aromatic substrates, chloride oxidation; Ullrich et al. 2004, 2008; Ullrich and Hofrichter 2007).

Due to the high degree of conservation of the heme-binding domain, it is conceivable that the conformation of the polypeptide chain near the cysteine ligand in AaP and CrP will be similar to that of CPO, forming a helix which is perpendicular to the heme stabilized by peptide amide–sulfur hydrogen bonds between the cysteine ligand and the surrounding residues (Sundaramoorthy et al. 1995). Such a helix starting with the cysteine was also calculated for the AaP sequence by phyre protein fold prediction (Bennett-Lovsey et al. 2008). However, based on an alignment of CPO and AaP sequences (Fig. 4), the substrate-binding as well as the distal heme pocket are seemingly different. In CPO, the proposed substrate-binding pocket is bracketed by the amino acid residues Phe_{103} and Phe_{186} (both also present in AaP, Phe_{121} and Phe_{199}). The heme is held in place by primarily backbone atoms interacting with the propionates of the heme molecule. The loop between Glu_{104} and Ser_{110} in CPO (Glu_{122} and Thr_{128} in AaP)

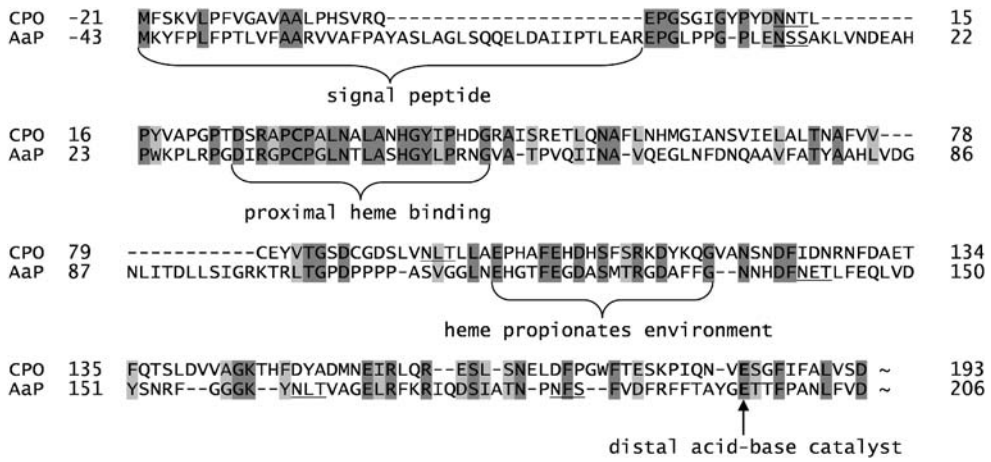

Fig. 4 Alignment of deduced protein sequences of *A. aegerita* peroxygenase (*AaP*) and chloroperoxidase (*CPO*). High homology of both sequences is particularly pronounced in the heme-binding region around the cysteine residue serving as axial heme ligand as well as, to some extent, in the region around the propionate residues of the heme and near the distal heme pocket. Hypothetically (AaP) and experimentally (CPO) determined *N*-glycosylation sites are *underlined*. Identical residues are shaded in *dark gray*. Chemically similar residues are shaded in *light gray*. Numbering starts with the first amino acid of the mature proteins. Homology seemingly ends after two third of the sequences, i.e., the C-termini of AaP and CPO differ considerably

provides the primary set of interactions and also contributes to the formation of the distal heme cavity together with a negatively charged glutamic acid residue at position 183 (position 196 in AaP). It was proposed that a positively charged histidine residue at position 105 in the distal heme cavity indirectly participates in the cleavage of the peroxide bond by hydrogen bonding to and correctly positioning of the direct acid–base catalyst, Glu_{183}, within the heme center (Sundaramoorthy et al. 1995). Although there is weak support (many gaps have to be introduced for this site in the alignment) for an acid–base catalyst glutamate at position 196 in AaP, a histidine residue at the comparable site in AaP is exchanged by a neutral glycine (Gly_{123}; Fig. 4) and therefore, AaP does not necessarily follow the same catalytical mechanism as CPO does.

Interestingly, AaP does not have cysteines at the comparable position—where CPO has a disulfide bridge (Cys_{79}–Cys_{87})—but a number of consecutive proline residues. In AaP, two cysteines at the C-terminal end (Cys_{278}–Cys_{319})—also present in constant distance and at conserved positions in the identified closely related hypothetical APO sequences (see below)—let us assume that they form a disulfide bridge in the mature protein. If so, the existence of a posttranslationally cleaved C-terminal peptide like in chloroperoxidase from *C. fumago* will be unlikely, as a cleavage in AaP after amino acid residue 301 (which is the last amino acid confirmed in protein sequence data) would inhibit disulfide bridging. Some of these assumptions may appear rather speculative; however, the sequences reported here will facilitate ongoing crystallographic studies to determine the tertiary structure of aromatic peroxygenase and to recognize the exact position of key residues responsible for its particular catalytical properties.

Expression studies on aromatic peroxygenase from A. aegerita According to earlier studies and unpublished data, soybean-based growth substrates are the most suitable media to induce peroxygenase production, not only in case of the fungal species tested here (*A. aegerita*, *C. radians*) but also in case of *C. verticillata, Agrocybe alnetorum*, *Marasmius* sp., and *Agaricus bisporus* (Ullrich et al. 2004; Anh et al. 2007; unpublished results). Other plant-based media (oat, potato, malt, tomato) only marginally promoted peroxygenase production, although enabling substantial fungal growth. Besides soybean flour, dried clover (*Trifolium* spp.) and alfalfa (*Medicago sativa*) leaves, as well as ground seeds from common beans (*Phaseolus vulgaris*), have been found to stimulate peroxygenase production in *C. radians* and to some extent also in *A. aegerita* TM-A1. These plants as well as the soybean (*Glycine max*) belong to the family of Fabaceae and are rich in organic nitrogen (proteins, peptides) and phenolic ingredients (flavonoides; Saloniemi et al. 1995; Wu et al. 2004). In the course of *A. aegerita* cultivation on soybean-based medium, the pH increased considerably (above pH 8), which was accompanied by the inset of APO secretion (Fig. 3c; Ullrich et al. 2004). This strong alkalization may be caused by the release of ammonium from soybean components (González et al. 2002). The pH increase alone, however, is not responsible for the enzyme induction (unpublished result). Thus, the actual induction of peroxygenase in agaric fungi depends on still unknown ingredients or compounds released from mentioned components of soybeans or other Fabaceae by the fungi. Therefore,

an important future objective will be the identification of the specific inducing agent(s) in soybean flour.

In order to answer the question when and how strong *apo*1 is induced in *A. aegerita* TM-A1, quantitative real-time PCR was performed and the peroxygenase activity was measured in parallel. Gene expression of *apo*1 was induced after a pH of 8 had been exceeded between the 14th and 19th day of cultivation and boosted afterwards till day 23. Enzyme activity always appeared later than the mRNA signal with an offset of about 7 days, which substantiates the fact that *apo*1 encodes the protein previously characterized as AaP (Ullrich et al. 2004).

It is conceivable that a low basal level of *apo*1 is always present in the fungi tested and hence a low APO activity as well. This may apply not just to *Agrocybe* and *Coprinellus* spp. but also to many other agaric fungi and even to other microbes. This admittedly daring hypothesis would explain why so many other fungal species code for APO-like proteins in their genomes or even transcribe *apo*-like mRNA (see below).

BLAST searches for nucleotide sequences Strong similarities of the peroxygenase sequences of AaP and CrP with putative protein sequences, provided through whole genome sequencing projects and transcriptome studies, were found in BLASTx searches. A substantial number of homologous genomic DNA sequences (over 100 sequences) from as many as 22 fungal species of 15 genera were retrieved. Surprisingly, these sequences do not only belong to related agaric species of *A. aegerita* and *C. radians*, such as *Coprinopsis cinerea*, but also to saprotrophic ascomycetes as *Chaetomium globosum, Podospora anserina*, and different *Aspergillus* species. Furthermore, the search hits comprise phytoparasitic fungi as the basidiomyceteous corn smut (*Ustilago maydis*), the ascomyceteous rice blast fungus (*Magnaporthe grisea*), and the potato blight fungus *Phytophthora infestans* (that is actually not a true fungus but a fungus-like heterokont). Finally, multiple APO homologous sequences are also present in the first fully sequenced ectomycorrhizal basidiomycete, *Laccaria bicolor*. Since it is unlikely that similar sequences have developed several times independently in the evolution, the occurrence of APO homologous sequences in taxonomically distant organisms points to a phylogenetically old protein (super) family.

The highest values of identity to AaP and CrP sequences (approximately 60%) were found for hypothetical protein

Table 3 Messenger RNA and EST sequences of putative heme-thiolate peroxygenases found throughout the fungal kingdom and beyond that

Class	Species	Strains	References and/or accession numbers
Agaricomycetes	*Agaricus bisporus*	D649	AJ293759
	Agrocybe aegerita	TM-A1	This study (FM872458)
	Amanita muscaria	No strain isoated *(Mycorrhiza with Populus tremula × P. tremuloides)*	AJ641329
	Coprinellus radians	DSMZ 888	This study (FM872459)
	Coprinopsis cinerea	Okayama7#130	DR752511
	Hebeloma cylindrosporum	Homocaryon h1+h7, dicaryon d2	Lambilliotte et al. 2004 (CK991695)
	Phanerochaete chrysosporium	Not mentioned	DV761776
	Pleurotus ostreatus	ASI2029	Lee et al. 2002 (AT004300)
Pucciniomycetes	*Phakopsora pachyrhizi*	Taiwan 72-1	Posada-Buitrago and Frederick 2005 (DN739810, DN739785, DN739461); EH238661
	Uromyces appendiculatus	Race41 (on *Phaseolus vulgaris*)	Cooper et al. 2007 (EH303709)
Eurotiomycetes	*Aspergillus nidulans*	FGSC A4	Brown et al. 1996 (ENU34740, stcC)
	Aspergillus niger	N400, N402, ATCC1015	Semova et al. 2006 (DR700090, DR701944); EY226567; EY242387
Dothideomycetes	*Caldariomyces fumago*	Not mentioned	Conesa et al. 2001 (AJ300448)
	Cochliobolus heterostrophus	C5	FK697079
	Phaeosphaeria nodorum	SN15	Hane et al. 2007 (EH399801, EH396465)
Sordariomycetes	*Gibberella zeae*	PH-1	BI750276, BI750029
	Podospora anserina	S mat+	Espagne et al. 2008 (CU887087, CU869645, CU870077)
Oomycetes	*Phytophthora capsici*	LT1534	BT031644, FG026369
	Phytophthora infestans	DDR7602	AF424685, AF424690
Mycetozoa (Myxogastrites)	*Hyperamoeba dachnaya*	Not mentioned	EC853454

sequences derived from genomic sequences of the agaric fungi *L. bicolor* and *C. cinerea*. However, we have to notice that despite the constant conservation of certain regions (in particular of the heme-binding domain), the homology of AaP and CrP to other sequences could be as low as to the sequence of CPO (27% identity, limited to the N-terminal part), thus indicating at least two groups of hypothetical peroxygenase-like proteins: proteins of the actual APO family (AaP, CrP, and the mentioned hypothetical proteins) and the CPO family (CPO and homologous hypothetical proteins).

Though the enzymatic proof of APO existence for these species is still pending, the hypothesis is supported by the evidence of transcribed APO-like and CPO-like genes. Apart from the known mRNA sequences of *A. aegerita*, *C. radians*, and *C. fumago*, a number of incomplete mRNA sequences from 17 species could be retrieved from data bases (Table 3). Using part of these sequences around and between the highest conserved regions (heme-binding and heme-propionate motifs), a multiple alignment was performed (Fig. 5). It appears that the sequence between the highly conserved domains of heme-binding and heme-propionate environments varies much and that again two groups of peroxygenase-like proteins can be distinguished. One group ("APO-like") is defined by high bootstrap values in phylogenetical analysis and comprises mRNA sequences of the known enzymes AaP and CrP as well as mRNA sequences of hypothetical proteins from *C. cinerea*, *Gibberella zeae*, and *Phaeosphaeria nodorum*. The other group ("CPO-like") is simply defined by nonmatching with the first group and contains just one characterized protein, CPO from *C. fumago*. In this context, it is remarkable that *P. nodorum* has sequences present in both groups, indicating that some organism may have the potential for expressing proteins of both peroxygenase-like families and that the existence of APO is not restricted to basidiomyceteous agarics. It is conceivable that more and more APO-like sequences will be found in fungi (and probably beyond that) in ongoing sequencing projects. Last but not least, heme-thiolate peroxidases as such, which have been an isolated position within in classification system of peroxidases so far, now appear in a different light and may be part of a larger protein superfamily.

Since there are strong indications for the existence of further aromatic peroxygenases in such different fungal or fungal-like phyla as basidiomycetes, ascomycetes, deuteromycetes, and oomycetes, the question arises: When and for which physiological and ecological functions the ancestral gene had been evolved in the evolution? At this time, we can only speculate whether the gene products are involved in the extracellular production of unspecific defense agents (e.g., hypohalides, organohalogens) or the modifi-

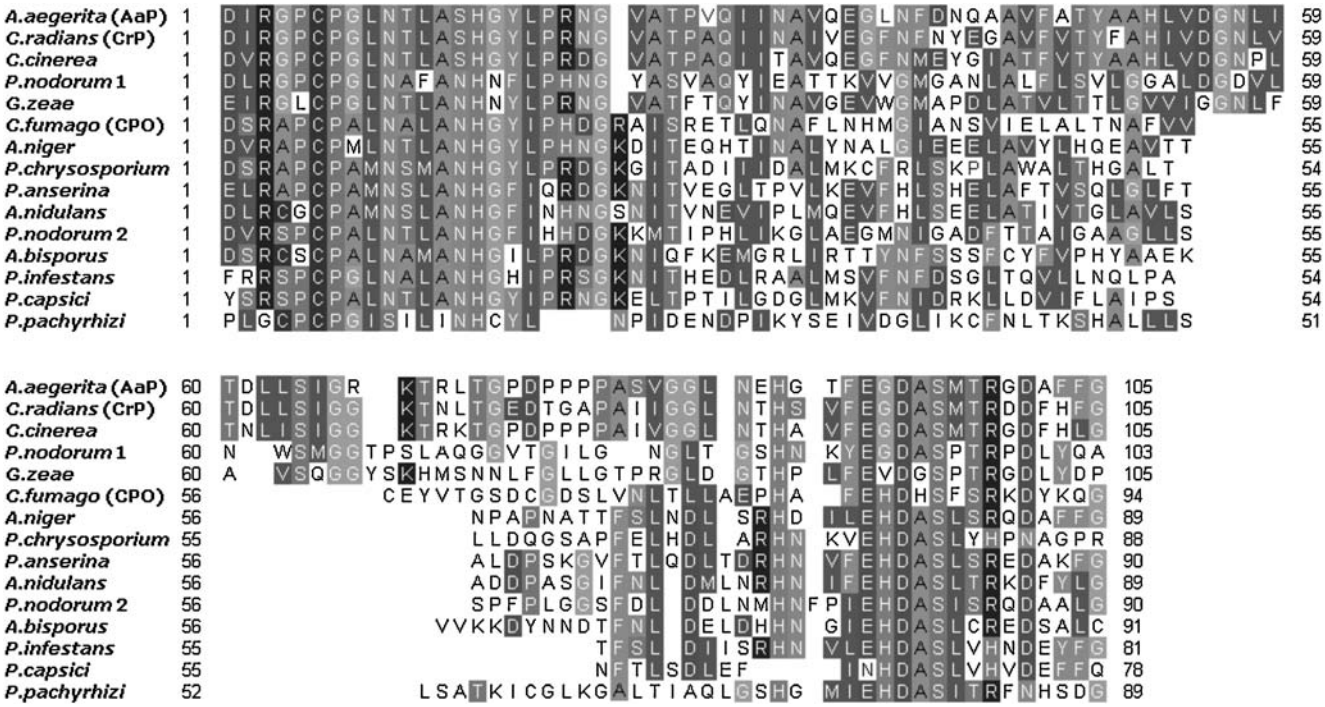

Fig. 5 Alignment of three true and 12 putative peroxygenase-like protein sequences derived from mRNA sequences of AaP, CrP, and CPO and data base matches. The latter matches are given as abbreviated scientific names of the fungi (for full names compare Table 3), from which the mRNA fragments were isolated. *AaP* and *CrP* stand for the purified and characterized aromatic peroxygenases of *A. aegerita* and *C. radians*; *CPO* chloroperoxidase from *C. fumago*

cation of recalcitrant biomolecules (oxygenation of aromatic compounds, e.g., lignin fragments). Taking into account the ability of AaP and CrP to oxidize and oxygenize a broad spectrum of recalcitrant compounds (polycyclic aromatics, heterocycles, phenolics, etc.), it appears reasonable that they act in both respect.

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