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Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes

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ABSTRACT

Extracellular peroxide generation, a key component of oxidative lignocellulose degradation, has been attributed to various enzymes including the copper radical oxidases. Encoded by a family of structurally related sequences, the genes are widely distributed among wood decay fungi including three recently completed polypore genomes. In all cases, core catalytic residues are conserved, but five subfamilies are recognized. Glyoxal oxidase, the most intensively studied representative, has been shown physiologically connected to lignin peroxidase. Relatively little is known about structure–function relationships among more recently discovered copper radical oxidases. Nevertheless, differences in substrate preferences have been observed in one case and the proteins have been detected in filtrates of various wood-grown cultures. Such diversity may reflect adaptations to host cell wall composition and changing environmental conditions.

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1. Introduction

Critical components of the carbon cycle, wood decomposing fungi have received increased attention for their potential in the production of biofuels and other bioprocesses. Most wood-decaying fungi are in the Agaricomycetes (Basidiomycota), although some species also occur in other groups of Basidiomycota and Ascomycota (Gilbertson, 1980; Nilsson et al., 1989; Shary et al., 2007; Wells and Bandoni, 2001). Two broad categories of wood decay chemistries are known in Agaricomycetes; white rot and brown rot. White rot fungi are uniquely capable of efficiently degrading and mineralizing all components of plant cell walls, including the highly recalcitrant lignin fraction. In contrast, brown rot fungi rapidly depolymerize cellulose but do not appreciably remove lignin, which remains as a polymeric residue (Blanchette, 1995; Eriksson et al., 1990; Niemenmaa et al., 2007; Worrall et al., 1997; Yelle et al., 2008). Resistant to further decay, brown rot residues contribute to the carbon pool in humic soils, particularly in conifer-dominated ecosystems. Evidence suggests that white rot is plesiomorphic in Agaricomycetes and that brown rot has evolved repeatedly (Floudas et al., 2012; Hibbett and Donoghue, 2001).

As described below, extracellular peroxide production plays an essential role in white rot and brown rot decay. Copper radical oxidases (CROs), the main focus of this review, likely fulfills this pivotal role.

2. Brown rot mechanisms

White and brown rot wood decay are complex and incompletely understood processes that have been intensively studied in the Polyporales *Phanerochaete chrysosporium* and *Postia placenta*, respectively. Other model white rot fungi include the polypores *Ceriporiopsis subvermispora, Bjerkandera adusta, Trametes versicolor* and the agaric *Pleurotus ostreatus*. The physiology of brown rot decay by *Gloeophyllum trabeum*, a member of the Gloeophyllales, has also received considerable attention.

Limited porosity is central to understand the mechanisms of wood degradation. Simply put, most enzymes are too large to penetrate sound, intact wood (Blanchette et al., 1997; Cowling, 1961; Flournoy et al., 1993; Srebotnik et al., 1988b; Srebotnik and Messner, 1991). Accordingly it has been proposed that enzyme-generated oxidizing species penetrate from the lumens into plant cell walls. Generated via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\cdot}OH$), hydroxyl radical has been strongly implicated as a diffusible oxidant in brown rot (Cohen et al., 2002; Xu and Goodell, 2001), and to a lesser extent, in white rot. Enzymatic saccharification of complex lignocellulose substrates is enhanced by hydroxyl radical pretreatment (Ratto et al., 1997), suggesting that brown rot involves sequential oxidation and hydrolysis.

The mechanisms controlling extracellular Fenton reactions are the subject of considerable debate (Baldrian and Valaskova,

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Table 1
Number of genes encoding select oxidoreductases in Agaricomcycete.[a]

Species	Order	GLX	CRO3-5	CRO6	CRO1	CRO2	LiP	MnP	VP	LAC	CDH	HTP	DYP	LPMO
F. pinicola	Polyporales	0	1	1	1	1	0	0	0	5	0	4	0	4
W. cocos	Polyporales	0	1	1	1	1	0	0	0	5	0	5	0	2
P. placenta	Polyporales	0	1	1	0	1	0	0	0	3	0	5	2	2
D. squalens	Polyporales	5	1	1	1	1	0	9	3	11	1	4	1	15
Ganoderma sp.	Polyporales	5	1	1	1	1	0	6	2	16	1	3	3	15
T. versicolor	Polyporales	5	1	1	1	1	10	13	2	7	1	3	2	18
P. brevispora	Polyporales	1	3	1	1	2	5	6	0	8	1	2	3	12
C. subvermispora	Polyporales	0	1	1	0	1	0	13	2	7	1	9	0	9
P.carnosa	Polyporales	1	2	1	1	1	4	7	0	0	1	3	0	11
P.chrysosporium	Polyporales	1	3	1	1	1	10	5	0	0	1	3	0	15
B. adusta	Polyporales	1	3	1	1	1	12	6	1	0	1	4	10	28
P. ostreatus	Agaricales	4	3	6	1	2	0	5	4	12	1	3	4	29
A. bisporus	Agaricales	3	1	1	2	2	0	2	0	12	1	24	0	11
S. commune	Agaricales	0	0	1	0	1	0	0	0	2	1	3	0	22
A. delicata	Auriculariales	2	1	2	0	3	0	5	0	7	1	16	11	19
S. lacrymans	Boletales	0	1	1	0	1	0	0	0	4	2	3	0	5
C. puteana	Boletales	0	0	1	1	4	0	0	0	6	1	2	0	10
P. strigosozonata	Corticiales	3	1	2	0	3	0	10	0	12	1	8	5	13
G. trabeum	Gloeophyllales	0	0	1	0	1	0	0	0	4	1	6	0	4
F. mediterranea	Hymenochaetales	0	1	1	1	1	0	16	0	10	1	4	3	13
H. annosum	Russulales	0	1	1	1	2	0	8	0	13	1	5	1	10
S. hirsutum	Russulales	3	1	1	0	3	0	10	0	15	1	10	2	16

[a] Limited to published genomes. All are wood decay species, except the compost-degrading button mushroom, *Agaricus bisporus* (Morin et al., 2012; Ohm et al., 2010). Sources include: (Floudas et al., 2012) for *Auricularia delicata*, *Dichomitus squales*, *Fomitiporia mediterranea*, *Punctularia strigosozonata*, *Stereum hirsutum*, *Trametes versicolor*; *Coniophora puteana*, *Fomitopsis pinicola*, *Gloeophyllum trabeum* and *Wolfiporia coccos*. See (Fernandez-Fueyo et al., 2012a) for *Ceriporiopsis subvermispora* (Olson et al., 2012) for *Heterobasidion annosum* (Martinez et al., 2009) for *Postia placenta* (Eastwood et al., 2011) for *Serpula lacrymans* (Ohm et al., 2010) for *Schizophyllum commune* and (Hori et al., 2013) for *Ganoderma* sp., *Bjerkandera adusta* and *Phlebia brevispora*. Abbreviations include: LiP, lignin peroxidase; MnP, manganese peroxidase; VP, versatile peroxidase; HTP, heme thiol peroxidase; DyP, dye-decolorizing peroxidases; LAC, laccase; GLX, glyoxal oxidase; CROs, copper radical oxidases most closely related to those of *P. chrysosporium* (Kersten and Cullen, 2013; Vanden Wymelenberg et al., 2006); CDH, cellobiose dehydrogenase; and LPMO, lytic polysaccharide monoxygenases formerly assigned to glycoside hydrolase family GH61. Gray shading highlights brown rot fungi.

2008; Goodell, 2003). Substituted hydroquinones are synthesized by several brown rot fungi and are thought to play a role (Cohen et al., 2004; Shimokawa et al., 2004). In *P. placenta*, the genes likely involved in hydroquinone biosynthesis, transport and reduction are upregulated in a medium containing ball milled aspen (BMA) as sole carbon source relative to glucose-containing medium. *P. placenta* tyrosinase- and laccase-encoding genes, neither having a *P. chrysosporium* ortholog, are also upregulated in BMA medium (Vanden Wymelenberg et al., 2010). Laccases have been identified in various white- and brown-rot fungi (Table 1) and may support a redox system via oxidation of hydroquinones (Gomez-Toribio et al., 2009; Guillen et al., 1997).

3. White rot mechanisms

Exclusively identified in white rot fungi, lignin peroxidase (LiP) and manganese peroxidase (MnP), their biochemistry (Higuchi, 1990; Kirk, 1988; Kirk and Farrell, 1987; Schoemaker and Leisola, 1990) and genetics (Alic and Gold, 1991; Cullen, 1997, 2002; Cullen and Kersten, 1996; Gold and Alic, 1993; Kersten and Cullen, 2007; Ruiz-Duenas et al., 2013) have been reviewed. LiP reactions include C_α–C_β cleavage of propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation, and aromatic cleavage of nonphenolic lignin model compounds (Hammel et al., 1985; Leisola et al., 1985; Renganathan and Gold, 1986; Renganathan et al., 1985; Tien and Kirk, 1984; Umezawa et al., 1986). MnP oxidizes Mn^{2+} to Mn^{3+}, using H_2O_2 as oxidant (Kuwahara et al., 1984; Paszczynski et al., 1985). Organic acids such as oxalic acid stimulate MnP activity by stabilizing the Mn^{3+}, and produce diffusible oxidizing chelates (Glenn et al., 1986; Glenn and Gold, 1985). MnP cleavage of the dominant non-phenolic structures within lignin may be mediated by lipid peroxidation mechanisms (Bao et al., 1994; Gutierrez et al., 2002; Kapich et al., 1999; Watanabe et al., 2000, 2001).

Designated "Versatile Peroxidases" (VPs), certain peroxidases oxidize Mn^{2+} as well as non-phenolic substrates (e.g. veratryl alcohol) in the absence of manganese (Camarero et al., 1999; Mester and Field, 1998). These enzymes typically feature Mn binding residues as well as a conserved Trp involved in the electron transfer that enables oxidation of non-phenolic compounds.

Deviations from the aforementioned peroxidase classifications have been observed (Fernandez-Fueyo et al., 2012a; Ruiz-Duenas

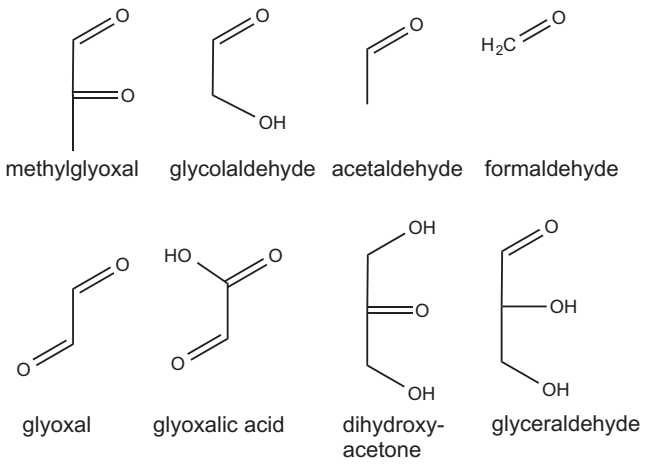

Fig. 1. GLX substrates. Aldehydes and α-hydroxycarbonyls are substrates for *P. chrysosporium* GLX (Kersten and Kirk, 1987). For simplicity, structures are represented in the unhydrated form.

et al., 2011). *C. subvermispora* protein models #118677 and #99382 were classified as LiP and VP genes, respectively, and the corresponding proteins are capable of oxidizing nonphenolic model compounds and synthetic lignin. However, the putative VP was unable to oxidize Mn^{2+}, and both enzymes showed catalytic properties intermediate between conventional LiPs and MnP (Fernandez-Fueyo et al., 2012a, 2012b).

Less well studied, but potentially involved in degradation of lignin and organopollutants, are heme thiolate peroxidases (HTPs) and dye decolorization peroxidases (DyPs) (Hofrichter et al., 2010; Lundell et al., 2010). HTPs include chloroperoxidases and peroxygenases, which catalyze a wide range of reactions including oxidations of various aliphatic and aromatic compounds (Gutierrez et al., 2011; Ullrich and Hofrichter, 2005). DyPs and putative DyP-encoding genes have been identified in various fungi, including white rot species (Table 1). High redox potential DyPs are attributable to the white-rot fungus *Auricularia auricula-judae* (Liers et al., 2010).

4. Peroxide generation

Central to Fenton reactions and to peroxidase catalysis, various extracellular systems for peroxide generation have been proposed. CROs (below) have been most intensively studied, but other oxidoreductases may also be involved.

Cellobiose dehydrogenase (CDH) contains a dehydrogenase domain and a heme prosthetic group (Hallberg et al., 2000). CDH oxidizes cellodextrins, mannodextrins and lactose, and suitable electron acceptors include quinones, phenoxyradicals, and Fe^{3+}. All known white rot genomes have a single CDH gene, but brown-rot genomes have none (*P. placenta*, *Fomitopsis pinicola*, *Wolfiporia cocos*), one (*Coniophora puteana, G. trabeum*), or two (*Serpula lacrymans*) gene copies (Eastwood et al., 2011; Fernandez-Fueyo et al., 2012a; Floudas et al., 2012). CDH is widely distributed among fungi (Table 1), but in view of its absence in *F. pinicola*, *P. placenta*, and *W. cocos,* the precise role remains uncertain (Henriksson et al., 2000; Zamocky et al., 2006). Suggesting an important role in cellulose degradation, co-expression of CDH and members of the GH61 family within the carbohydrate-active enzyme database (Lombard et al., 2014) has been observed. Now classified as copper-dependent lytic polysaccharide monooxygenases (LPMOs) (Quinlan et al., 2011; Westereng et al., 2011), GH61s boost cellulose depolymerization by CDH (Harris et al., 2010; Langston et al., 2011).

Other enzymes implicated in peroxide generation include methanol oxidase (Daniel et al., 2007), pyranose oxidase (Daniel et al., 1994), and aryl alcohol oxidases (Hernandez-Ortega et al., 2012). The latter enzyme is widely distributed in white and brown rot fungi and is simultaneously expressed with class II peroxidases in *B. adusta* and *Pleurotus* cultures (Camarero et al., 1996; Hernandez-Ortega et al., 2012). In addition to MnPs, recent mass spectrometry analysis of *C. subvermispora* grown on ground wood identified both aryl alcohol oxidases and CROs (Hori et al., 2014).

5. Glyoxal oxidase

5.1. Physiological context in P. chrysosporium

Glyoxal oxidase (GLX) was discovered in a search for extracellular peroxide that would support peroxidase activity in ligninolytic cultures of *P. chrysosporium* (Kersten and Kirk, 1987). These cultures were nutrient-nitrogen limited with glucose as carbon source. No peroxide generating activity was observed in these cultures with cellobiose, glucose, or xylose as substrates and therefore a broader net of possible substrates was cast; the rational was that if primary wood sugars were not substrates, then a metabolic product(s) might serve as the electron source for the reduction of molecular oxygen. Peroxide-generating activities with simple aldehyde and hydroxy-carbonyl substrates was observed with culture fluid (see Fig. 1 for structures).

The non-specificity of the enzyme activities presented a challenge for the identification of a physiological substrate. Most of the identified substrates produce bis-derivatives with 2,4-dinitrophenylhydrazine which precipitate from acidic aqueous solution allowing simple purification and identification by thin layer chromatography. Hence, bis-derivatives of methylglyoxal and glyoxal were identified, but the analytical method presents ambiguity; glycolaldehyde gives the same bis-derivative as glyoxal, whereas glyceraldehyde, dihydroxyacetone, and acetol give the same bis-derivative as methylglyoxal. Nevertheless, this provided a first rapid screening to test whether potential substrates could be in culture. More specific analytical techniques identified both glyoxal and methylglyoxal. Accordingly, the enzyme with the newly discovered activity was named glyoxal oxidase (GLX). Importantly, the appearance of both GLX and its substrates were coincident with lignin peroxidase during secondary metabolism (Kersten and Kirk, 1987).

To elucidate the contributions of the various substrates of GLX in peroxide production, the steady-state kinetic parameters for GLX were determined with a lignin peroxidase coupled assay (Kersten, 1990). The second-order rate constants (k_{cat}/K_m) indicated that methylglyoxal was the best substrate, with glyoxal at 3.3% relative activity. However, the k_{cat} values with the various substrates were relatively constant but K_m values varied widely indicating that rates of reaction may be controlled at the substrate concentration level. Furthermore, the second-order rate constants for glycolaldehyde and glyoxylic acid were better than for glyoxal, suggesting efficient sequential oxidations (Kersten, 1990). This concept was pursued in coupled reactions with lignin peroxidase and a β-aryl ether lignin substructure model compound as peroxidase substrate (Hammel et al., 1994). Glycolaldehyde is a product from the lignin substructure and serves as substrate for GLX for sequential reactions (glycolaldehyde → glyoxal → glyoxylic acid → oxalate), thus supplying peroxide that may be recycled to the lignin peroxidase. Yet another possible source of glyoxal in cultures is the MnP-dependent lipid peroxidation of linoleic acid, a fungal metabolite (Watanabe et al., 2001). Carbohydrate fragmentation by hydroxyl radical is also

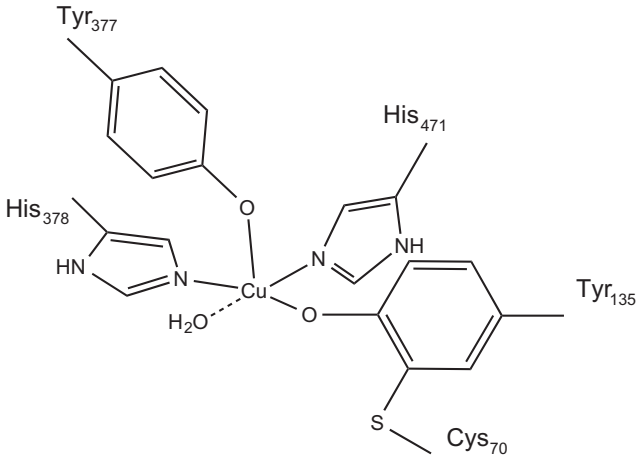

Fig. 2. Active site of *P. chrysosporium* GLX based on GAO, adapted from (Whittaker et al., 1999). The active site incorporates amino acid residues from remote regions of the polypeptide. Tyr135, Tyr377 and His378 derive from the first domain that has a superbarrel structure (see Fig. 3). The Tyr135 is covalently linked to Cys70 by a thioether bond. The His471 is derived from the C-terminal domain.

likely to supply GLX substrates; abiotic oxidation of sugars and trioses in a Fenton system produces glyoxal (Manini et al., 2006). Both glyoxal and methylglyoxal have long been recognized as products of glucose auto-oxidation in the presence of proteins or amino acids (Thornalley et al., 1999).

A notable feature of native GLX is that it is reversibly inactivated by purification, or even by simple dialysis, as monitored by oxygen consumption in the absence of a coupled peroxidase (Kersten, 1990). GLX is reactivated by LiP and veratryl alcohol, a metabolite synthesized by *P. chrysosporium*. This modulation of activities by interaction between oxidase and peroxidase systems may be physiologically significant, allowing the production of peroxide only when a suitable peroxidase substrate is present. The cloning and heterologous expression of recombinant GLX, rGLX (Kersten and Cullen, 1993; Kersten et al., 1995) allowed more detailed studies of the activation and inactivation of rGLX (Kurek and Kersten, 1995). Results showed that peroxidase substrates of high redox potential were effective activators, whereas phenolics inactivated rGLX. Either LiP or horseradish peroxidase (HRP) could be used in the activation, but only if a suitable high redox potential peroxidase substrate was included. Lignin itself was an effective activator in this system.

5.2. Catalytic mechanism – similarity to galactose oxidase

The *P. chrysosporium* GLX is a CRO, or radical copper oxidase (Whittaker, 2002; Whittaker et al., 1999, 1996), a metalloenzyme combining two distinct redox centers, one a copper metal ion, the other a stable protein free radical. Galactose oxidase (GAO) from *Fusarium* sp. is the best characterized copper radical oxidase for which crystal structure (Ito et al., 1994) and detailed biochemical characterizations are reported (see review (Whittaker, 2005)). Although crystal structure is not available for GLX, spectroscopic analyses of native and mutant GLX proteins show that the active sites of GLX and GAO are remarkably similar, despite less than 20% amino acid sequence similarity between proteins (Whittaker et al., 1999, 1996). However, the two enzymes are distinct in substrate specificity, redox potential, stability of the protein free radical, and number of domains. The summary here focuses on GLX and relies heavily on spectroscopic comparisons with GAO, together with mutational analyses, to determine the catalytic mechanism and the identification of active-site amino acids (Whittaker et al., 1999, 1996).

GLX catalyzes the two-electron oxidation of simple aldehydes and hydroxycarbonyls with the reduction of molecular oxygen to hydrogen peroxide (Whittaker, 2005). Although this is a two-electron redox reaction, spectroscopic analyses of the active site show that the catalysis involves two distinct redox centers, and electrons are transferred one electron at a time.

$$RCHO + O_2 \rightarrow RCO_2H + H_2O_2$$

The unique active site of *P. chrysosporium* GLX incorporates amino acid residues from remote regions of the polypeptide creating four copper metal binding residues, Tyr135, Tyr377, His378 and His471. Of particular interest, the Tyr135 is covalently linked to Cys70 by a thioether bond (Fig. 2). Sequence alignments indicate a histidine overlying this Tyr-Cys dimer in contrast to tryptophan in GAO, which may strongly contribute to key differences in the enzymes. The unmodified Tyr377 appears to serve in general base catalysis, activating the substrate for oxidation (Whittaker et al., 1999). The Cu(II) enzyme, or resting enzyme, is catalytically inactive. When the enzyme is oxidized by one electron, or the copper reduced by one electron, the enzyme enters the catalytic cycle (Whittaker et al., 1999). Therefore, the regulation of GLX activity by LiP has an explanation on the molecular level, with the oxidizing intermediates generated by LiP activating resting GLX.

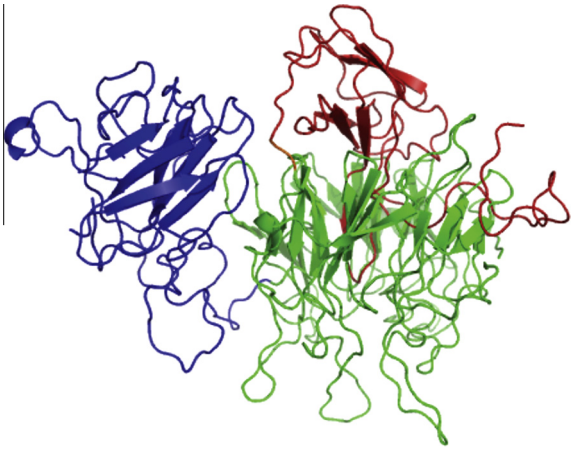

Fig. 4. Protein model of *P. chrysosporium* CRO6. The predicted Phyre2 model of CRO6 indicates an N-terminal domain (blue) with similarity to putative xylan esterase axe2 of *Cellvibrio japonicas*, whereas the second (green) and third (red) domains with the second and third domains of GAO template.

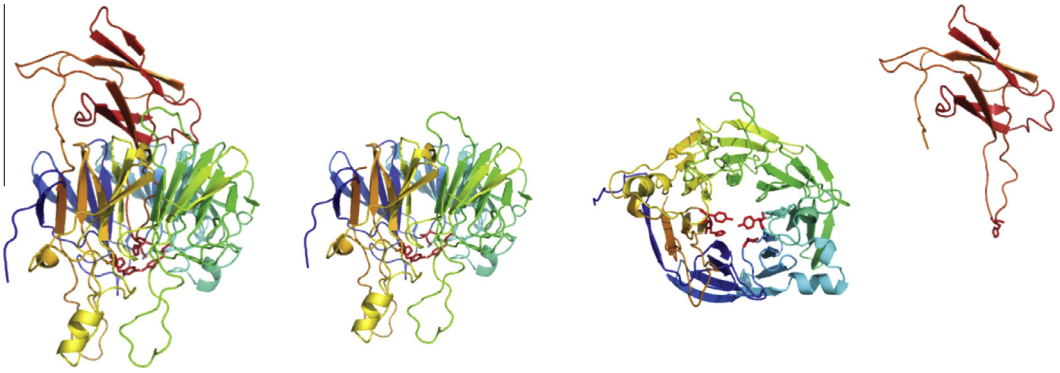

Fig. 3. Protein model of *P. chrysosporium* GLX. The PyMOL representation of GLX (Phyre2 model) is shown in rainbow-spectrum cartoon mode with the active site residues (see Fig. 2) in red stick mode. At left is the representation of the two-domain structure. For clarity, the first domain is also shown alone in the same orientation, and then rotated 90 degrees, thus making Cys70, Tyr135, Tyr377 and His378 more visible. At far right is the second domain with the loop that extends down the center of the superbarrel providing His471 ligand of the copper protein.

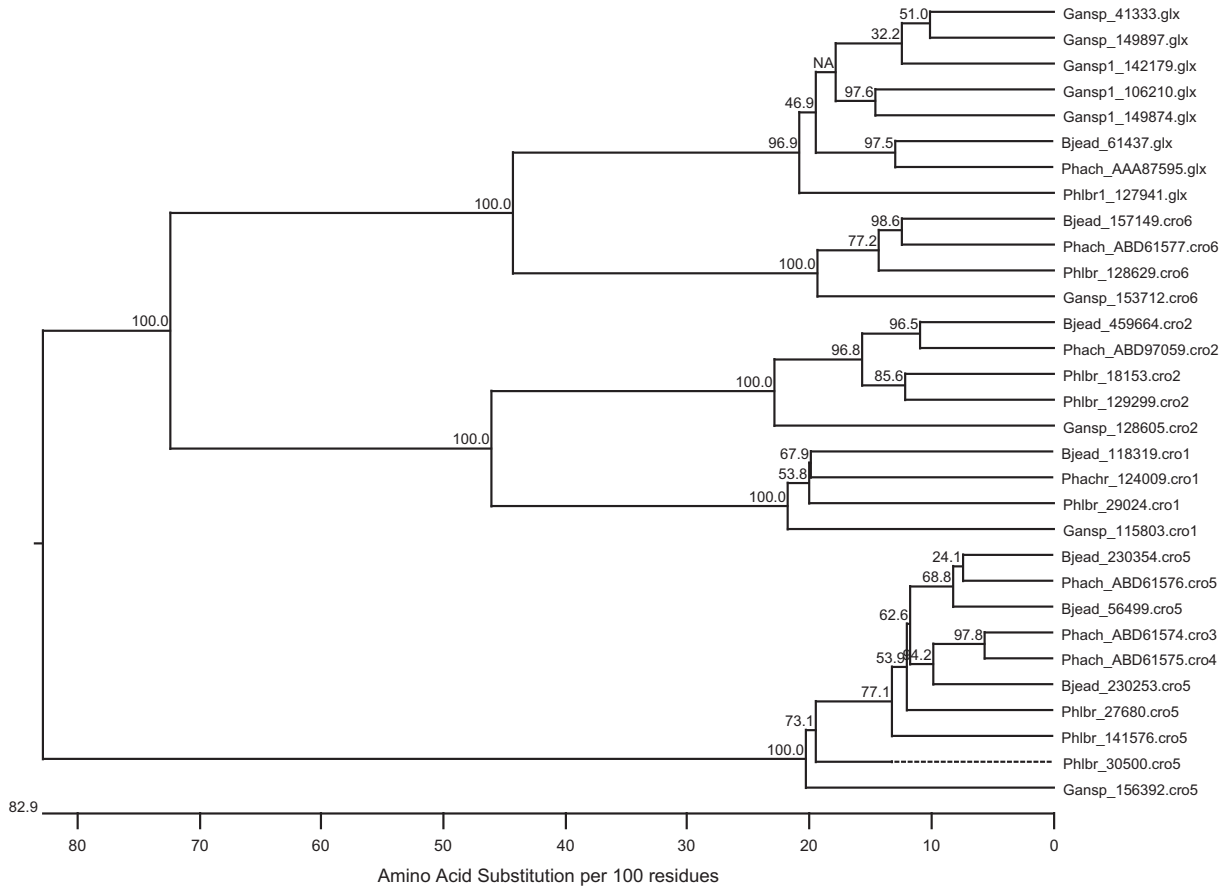

Fig. 5. Relationships between predicted CRO proteins of *P. chrysosporium* (Phach) and recently sequenced polypores *Ganoderma* sp. (Gansp), *B. adusta* (Bjead) and *P. brevispora* (Phlbr) (Hori et al., 2013). Clustal W alignments and tree constructions were performed following manual trimming of secretion signals. Bootstrap values (1000 trials) are shown at nodes and the Kimura distance formula used to calculate substitutions.

The active sites of GLX and GAO are housed in domains showing a 7-fold β-barrel structure, or "super-barrel" (Bork and Doolittle, 1994). In GLX, this is the first domain, and provides Cys70, Tyr135, Tyr377 and His378 to the active site. A second domain caps the superbarrel providing His471 at the end of a loop extending down the center of the superbarrel (Whittaker et al., 1999). Here we illustrate the predicted tertiary structure of GLX in Fig. 3 based on Phyre2 modeling (Kelley and Sternberg, 2009) with GAO as template and visualized with PyMOL Molecular Graphics System software, Version 1.5.0.4 Schrödinger, LLC.

6. Other CROs

Following the initial characterization of the *P. chrysosporium* cDNA (Kersten and Cullen, 1993) encoding GLX, genome analyses identified five subfamilies, all with conserved catalytic residues (Vanden Wymelenberg et al., 2006). Designated CRO1, CRO2, CRO3, CRO4, CRO5 and CRO6, the *P. chrysosporium* genes featured conserved catalytic residues. Constituting a single, highly conserved subfamily, CRO3, CRO4 and CRO5 all feature tandem copies of a highly conserved WSC domain (IPR002889) at their 5′ termini, and the three are clustered within a larger cluster of LiP genes. Widely distributed in functionally unrelated proteins, the WSC domain may be involved in carbohydrate binding. A 3′ terminus transmembrane helix in CRO2 is also present in the CRO2 homolog of the corn smut pathogen, *Ustilago maydis* glo1. The latter gene has been linked to filamentous growth and pathogenicity (Leuthner et al., 2005). Aldehyde substrate preference of *P. chrysosporium* CRO2 and GLX differ substantially (Vanden Wymelenberg et al., 2006). CRO6 is predicted to have an N-terminal domain preceding the superbarrel CRO domain (Fig. 4).

The number and family distribution of CROs vary among Agaricomycetes, although relationship(s) to ecology and taxa are unclear. Our analysis of recently sequenced polypores (*Ganoderma* sp., *Phlebia brevispora, Bjerkandera adusta*) assigns at least one protein to each family (Fig. 5) with five GLX representatives identified in *Ganoderma* sp. (Table 1). Distant linkage was detected between the *Ganoderma* sp. GLX genes and for three *B. adusta* CRO5-like genes. GLX homologs are lacking from brown rot fungi and, consistent with a role in peroxidase oxidation, found only in genomes with LiPs and/or MnPs. However, these Class II peroxidases are present in white rot species *C. subvermispora, F. mediterranea* and *H. annosum*, all of which lack GLX (Table 1). Possibly, other CROs such as the WSC-containing CRO3–CRO5 compensate by oxidizing an array of metabolites unique to *C. subvermispora, F. mediterranea* and *H. annosum*. In contrast, the white rot fungus *S. commune*, lacks ligninolytic peroxidases and most CROs. In line with this gene complement, *S. commune* is limited in its ligninolytic ability (Schmidt and Liese, 1980).

CRO genes are differentially expressed in response to media composition. Coordinate with ligninolytic peroxidases, GLX transcript and extracellular peptides are upregulated in nutrient limited *P. chrysosporium* cultures (Vanden Wymelenberg et al., 2009). Elevated transcript levels of the *C. subvermispora cro2* gene were observed in wood-containing medium, and peptides corresponding to CRO5 were detected in medium using microcrystalline cellulose as sole carbon source (Fernandez-Fueyo et al., 2012a).

CRO5 proteins have also been detected in extracellular filtrates of BMA media inoculated with *T. versicolor*, *A. delicata*, and *D. squalens* (Floudas et al., 2012), and more recently in *Ganoderma* sp., *P. brevispora* and *B. adusta* cultures supplemented with Wiley mill ground aspen (Hori et al., 2014). The CRO2 proteins have been identified in eight white rot genomes and 4 brown rot genomes when the fungi are grown on BMA. Under these conditions, detection of CRO1 and CRO6 peptides has been limited to *Dacrypinax* sp. and *A. delicata*, respectively (Floudas et al., 2012). Earlier reports showed that the substrate preference of *P. chrysosporium* CRO2 differed sharply from GLX (Vanden Wymelenberg et al., 2006).

7. Current research and future prospects

A physiological role for GLX can be supported from several lines of evidence; (1) GLX produces peroxide required by peroxidases, (2) peroxide-generation by GLX is modulated by peroxidases, and (3) GLX substrates may be derived from the fragmentation of lignocellulosics, both lignin and carbohydrate components. Together with transcriptomic and proteomic data, this suggests a role for GLX in peroxide production for diverse oxidative reactions during wood decay by Agaricomycetes. The functions of other CROs are less certain. CRO2 for example, in comparison to GLX, appears more limited in substrate specificity, preferentially oxidizing glycolaldehyde. However, substrate specificity by itself may not be a good indicator of function for CROs. For example, the canonical substrate for galactose oxidase (i.e. galactose) is not the best substrate, instead, dihydroxyacetone is preferentially oxidized (Zancan and Amaral, 1970). Substrate specificity of other CROs is unknown and points to an area of needed research. Also, predicted protein structures of CROs indicate probable differences in physiological roles. CRO2 of *P. chrysosporium,* for example, has a C-terminal transmembrane region, depending on transcript processing, suggesting a role distinct from that of GLX. The functions of the N-terminal WSC domains of CROs3-5 remain unclear, and the homology of the N-terminal domain of CRO6 of Agaricomycetes suggests an important conserved function. In this context, encouraging progress has been made in the development of techniques for targeted gene replacement (Salame et al., 2013, 2012, 2011) for the white rot fungus *Pleurotus ostreatus*. Such genetic 'toolboxes' may help establish the function of genes encoding copper radical oxidases.

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