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Fungal PQQ-dependent dehydrogenases and their potential in biocatalysis

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In 2014, the first fungal pyrroloquinoline-quinone (PQQ)-dependent enzyme was discovered as a pyranose dehydrogenase from the basidiomycete *Coprinopsis cinerea* (*Cc*PDH). This discovery laid the foundation for a new Auxiliary Activities (AA) family, AA12, in the Carbohydrate-Active enZymes (CAZy) database and revealed a novel enzymatic activity potentially involved in biomass conversion. This review summarizes recent progress made in research on this fungal oxidoreductase and related enzymes. *Cc*PDH consists of the catalytic PQQ-binding AA12 domain, an N-terminal cytochrome *b* AA8 domain, and a C-terminal family 1 carbohydrate-binding module (CBM1). *Cc*PDH oxidizes 2-keto-D-glucose (D-glucosone), L-fucose, and rare sugars such as D-arabinose and L-galactose, and can activate lytic polysaccharide monooxygenases (LPMOs). Bioinformatic studies suggest a widespread occurrence of quinoproteins in eukaryotes as well as prokaryotes.

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Introduction

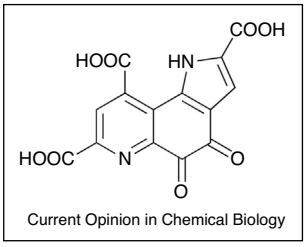
Quinoprotein is a general term used for enzymes containing an ortho-quinone or para-quinone as a prosthetic group. Pyrroloquinoline quinone (PQQ; Figure 1) has been referred to as the third redox cofactor [1,2] following nicotinamide pyridine nucleotide ($NAD(P)^+$) and flavin (FAD, FMN). Next to PQQ, other quinone prosthetic groups include topa quinone (TPQ) [3], tryptophan tryptophylquinone (TTQ) [4], lysine tyrosylquinone (LTQ) [5], and cysteine tryptophylquinone (CTQ) [6,7]. PQQ is tightly, but not covalently, bound to the enzyme, primarily via electrostatic interactions, whereas other quinones are formed by posttranslational modifications of amino acid residues and are thus covalently attached to the enzyme [8]. PQQ quinoproteins are known from prokaryotes where they primarily catalyze the dehydrogenation of the primary or secondary hydroxyl group of alcohols or sugars [9–11]. Some of these PQQ-dependent enzymes also contain one or more heme groups and are called quinohemoproteins [12,13]. A few bacterial species such as methylotrophic bacteria are able to synthesize PQQ, whereas non-PQQ-synthesizing bacteria such as *Escherichia coli* rely on the environment for its supply [14].

Although PQQ has nutritional importance and pharmacological effects in higher organisms, including humans [15], until recently, there was no clear evidence for the use of PQQ as a co-factor in a eukaryotic enzyme. This review presents a novel eukaryotic PQQ-dependent quinohemoprotein recently discovered from the saprophytic basidiomycete *Coprinopsis cinerea*, with a substrate specificity not previously described for fungi, and with possible links to fungal biomass conversion.

A new group of PQQ-dependent enzymes

Cellulolytic fungi are important decomposers of plant biomass and secrete not only a set of glycoside hydrolases but also numerous oxidoreductases, including cellobiose dehydrogenases (CDHs) and lytic polysaccharide monooxygenases (LPMOs), for the degradation of plant cell wall polysaccharides [16,17••]. In the database of Carbohydrate-Active enZymes (CAZy, http://www.cazy.org/), these oxidoreductases are categorized into Auxiliary Activities (AAs) families, which comprise redox enzymes that act in conjunction with other CAZymes [18]. Family AA12 was established in September 2014 upon the discovery of a secreted PQQ-dependent pyranose dehydrogenase,

Figure 1



Pyrroloquinoline quinone (PQQ).

derived from *C. cinerea* (*Cc*PDH). *Cc*PDH is composed of an N-terminal cytochrome domain belonging to the AA8 family, the AA12 catalytic domain and a C-terminal cellulose-binding domain belonging to carbohydrate-binding modules (CBMs) family 1, as shown in Figure 2 [19••]. The existence of CBM1 indicated the role of this enzyme in plant cell wall degradation, and thus led the dehydrogenase domain of *Cc*PDH to be introduced into AA class as a new family. The recombinant protein heterologously expressed in *Pichia pastoris* showed oxidative activity towards various sugars (see below) in the presence of PQQ and calcium ion. *Cc*PDH is the first PQQ-dependent enzyme found in eukaryotes.

BLAST searches showed that the amino acid sequence of the AA12 domain in *Cc*PDH is not similar with those of known prokaryotic PQQ-dependent enzymes, indicating that AA12 enzymes indeed represent a new family of quinoproteins. Genes encoding homologues of the PQQ-binding dehydrogenase domain of *Cc*PDH are found in many fungal genomes. The fungal AA12 homologues show four types of domain structures, single AA12 domain (majority), AA12-CBM1, AA8-AA12, and AA8-AA12-CBM1 signal peptide, as shown in Figure 3. Interestingly, AA12 homologues were also found in bacterial, archaeal, and amoebozoal genomes, and all these enzymes show single domain structures [19••]. The fact that non-fungal AA12 homologues are also PQQ-dependent enzymes was confirmed by the study of an AA12-homologue from the bacterium *Pseudomonas aureofaciens* (named *Pa*2KGDH) [20•]. This study showed that *Pa*2KGDH specifically oxidizes the C1-position of 2-keto-D-glucose (2KG; also known as D-glucosone), and only in the presence of PQQ, producing 2-keto-D-gluconic acid (2KGA), an activity that is also displayed by *Cc*PDH, as outlined below. This indicates that PQQ-dependent AA12 enzymes, with primary sequences that are remarkably distinct from those of previously characterized prokaryotic PQQ-dependent enzymes, are widely distributed, from prokaryotes to eukaryotes.

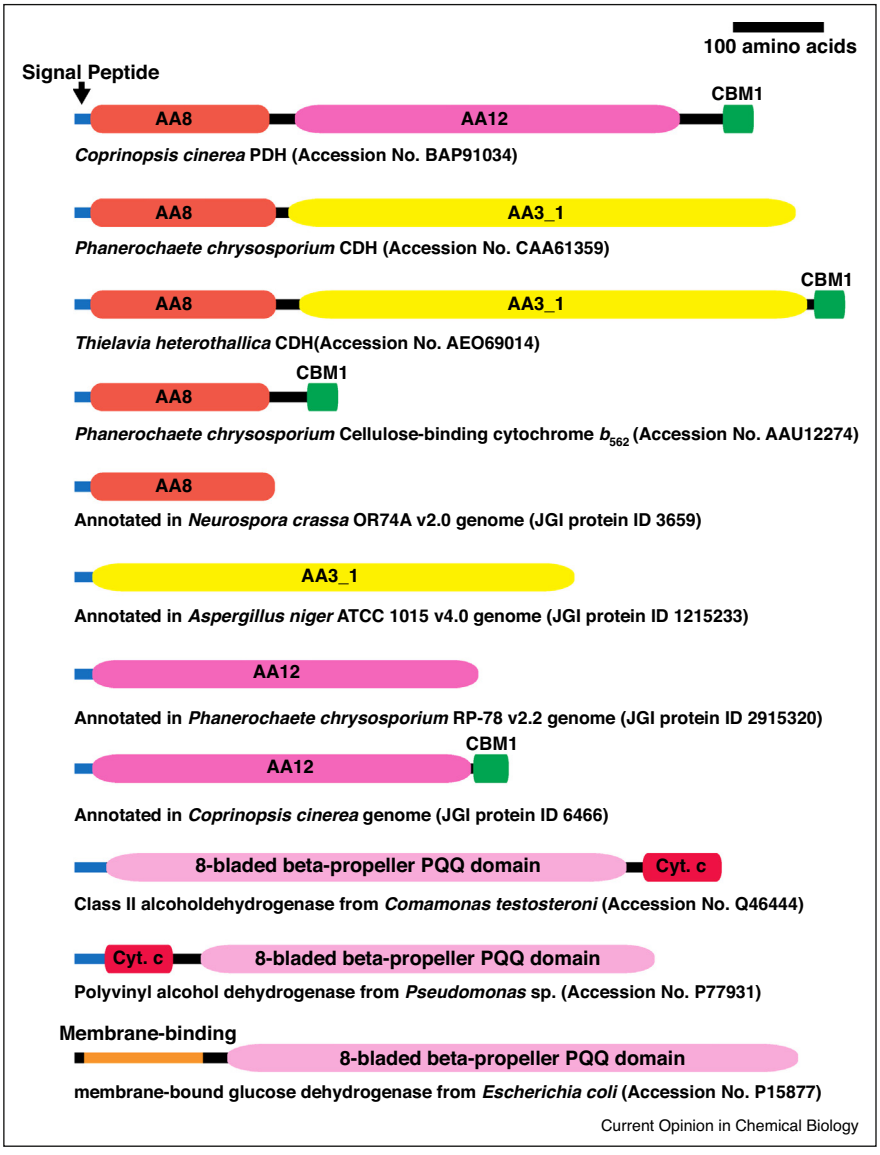
*Cc*PDH, a fungal PQQ-dependent enzyme

The PQQ and heme *b* cofactors of *Cc*PDH are located in the 45 kDa AA12 and the 21 kDa AA8-cytochrome domains, respectively, and these domains are connected by a proline-rich linker region. The domain organization of *Cc*PDH resembles those of some CDHs, which have a flavin-containing AA3 domain instead of the PQQ-containing AA12 domain (Figure 2). This domain organization is unique for PQQ enzymes, as only a *c*-type cytochrome domain and membrane-binding domain have been identified in known PQQ enzymes so far. *Cc*PDH showed PQQ-dependent catalytic activity, as demonstrated by the increase in activity observed upon increasing the PQQ concentration up to stoichiometric amounts. Isothermal titration calorimetry data demonstrated that the AA12 domain binds PQQ with a 1:1 stoichiometry, and has strong affinity for the co-factor, with a dissociation constant, K_d, of 1.1 nM [21••]. Most recently, we determined the crystal structure of the AA12 domain of *Cc*PDH, which provides structural evidence for binding of PQQ to the protein (Takeda *et al.*, unpublished results). The structure shows that PQQ is bound at the active site together with a catalytically essential calcium ion.

The co-occurrence of the AA12 and the AA8 domains in *Cc*PDH would allow for electron transfer between these domains. Such electron transfer is known to occur in CDH, and the AA8 family in CAZy is in fact largely comprised of cytochrome domains of multi-domain CDHs. One notable exception concerns the carbohydrate-binding cytochrome b_{562} from *Phanerochaete chrysosporium*, a two-domain structure consisting of an AA8 and a CBM1 but not containing a dehydrogenase domain [22]. AA8 domains contain a 6-coordinated low-spin heme *b* group within an ellipsoidal antiparallel β-sandwich fold [23]. The heme is bound in a hydrophobic pocket at one face, with one heme edge being exposed to the solvent, and being axially ligated by Met and His. The amino acid sequence of the AA8 domain in *Cc*PDH has 32–42% sequence identity with the AA8 domains of basidiomycete CDHs. The UV–vis and resonance Raman spectra of *Cc*PDH in the oxidized and reduced forms are in good agreement with spectra obtained for the AA8 domain of *P. chrysosporium* CDH (*Pc*CDH) [21••]. Our electrochemical studies indicated that the redox potential of the heme is around +130 mV versus NHE at pH 7.0, which is almost identical to the value obtained for *Pc*CDH [21••].

Plant cell wall degrading enzymes often contain a non-catalytic CBM. The majority of CBMs attached to fungal cellulolytic enzymes belongs to family 1. The presence of a CBM1 indicates a role in plant cell wall degradation, as the main function of CBM1s is to facilitate binding of the enzyme to the surface of cellulose, which is thought to enhance catalytic efficiency by increasing the effective enzyme concentration near the substrate. Some ascomycetous CDHs possess a CBM1 at their C-terminus, whereas basidiomycetous CDHs lack this domain [24]. The C-terminal CBM1 of *Cc*PDH contains three conserved aromatic residues that likely contribute to binding on

Figure 2



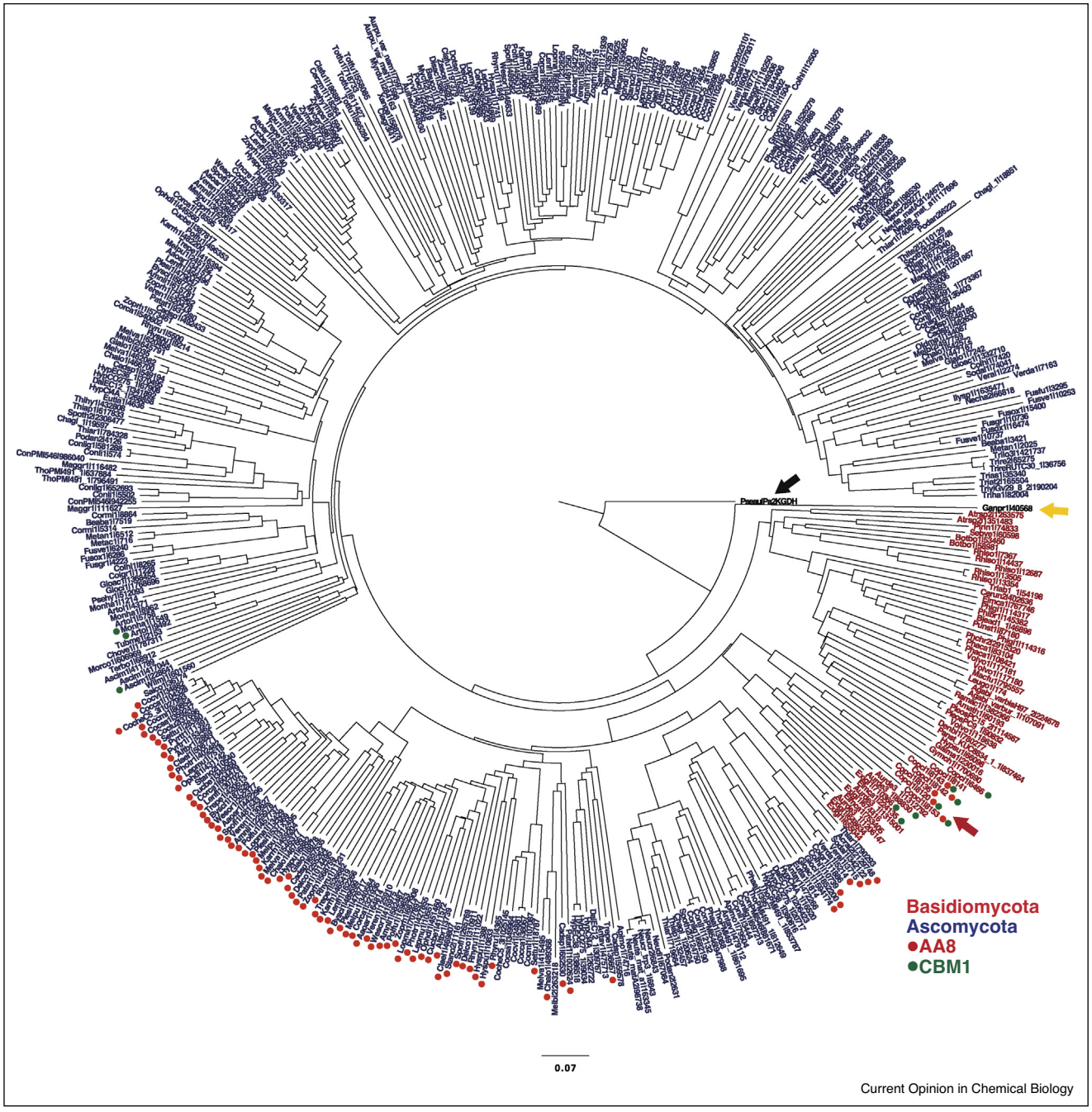
Domain organization of *Cc*PDH and related proteins.
Abbreviations: AA3_1, Auxiliary Activities (AA) family 3 subfamily 1 domain [a flavoprotein containing a flavin-adenine dinucleotide (FAD)]; AA8, AA family 8 domain, a *b*-type cytochrome; AA12, AA family 12 domain (a PQQ-dependent dehydrogenase domain); CBM1, family 1 carbohydrate-binding module; Cyt. c, *c*-type cytochrome domain.

cellulose, as suggested by in-depth studies of the functionality of other CBM1 domains [16]. Because *Cc*PDH shows high affinity toward microcrystalline and amorphous celluloses [21••], in its natural environment, the enzyme presumably is localized on the surface of cellulose.

Substrate specificity

*Cc*PDH is special; in that, it has broad substrate specificity and, thus, is a versatile biocatalyst. In our first report on this enzyme [19••], it was named sugar dehydrogenase (*Cc*SDH) because of its oxidizing activity toward various sugars. It was renamed pyranose dehydrogenase (*Cc*PDH) when the enzyme was found to act on a wide variety of pyranoses [21••]. *Cc*PDH shows significant activity toward 2KG, L-fucose, and rare sugars such as D-arabinose, L-galactose, D-talose, and L-gulose, and substrate oxidation at the C-1 position was confirmed by using L-fucose as a substrate [25•]. Thus, *Cc*PDH seems to prefer the $^{1}C_{4}$ conformation of aldose sugars with C-2 and C-3 hydroxyl groups in an equatorial configuration and the C-4 hydroxyl group in an axial configuration, as suggested in a previous study [21••]. Regarding 2KG, the $^{1}C_{4}$ conformation is one of

Figure 3



Phylogenetic tree of fungal AA12 domains.
The phylogenetic tree was generated with the amino acid sequences of AA12 domains registered in the Joint Genome Institute MycoCosm database (https://genome.jgi.doe.gov/programs/fungi/index.jsf). The sequences obtained were subjected to alignment analysis using MAFFT (ver. 6.85). Non-conserved regions, including AA8 and CBM1 domains, were trimmed using SeaView (ver. 4.4.2) software. The phylogenetic tree of the AA12 domains was generated from the trimmed sequences using ClustalX (ver. 2.1) with the neighbor-joining method (Bootstrap value, 1000). The red arrow indicates *Cc*PDH. The yellow arrow indicates AA12-homologue from *Gonapodya prolifera*, which is the only Chytridiomycotous enzyme found in the database. The black arrow indicates *Pa*2KGDH, the only bacterial AA12 used in this phylogenetic analysis.

the more plausible tautomers, at 21% abundance in aqueous solution [26], but the C-1 position of this conformation is clearly different from those of other pyranoses such as L-fucose, D-arabinose, and L-galactose. Moreover, it is worth noting that the affinity of *Cc*PDH for the substrates is quite low, with K_m values in the mM range. Therefore, while there is a clear biocatalytic potential here, the mechanism of substrate recognition in this enzyme is still unclear, and more studies are needed to clarify the details of its catalytic action.

As described above, *Cc*PDH acts on various pyranoses including rare sugars, and recently we found that the enzyme can oxidize the C-1 position of L-guluronate, which is an acidic sugar of L-gulose [27]. The substrate specificity of *Cc*PDH is clearly different from those of previously characterized prokaryotic PQQ-dependent enzymes, as shown in Table 1. The unique broad substrate specificity of *Cc*PDH can be used in biorefinery processes, especially to convert rare sugars and uronates to corresponding oxidized forms. In fact, we are currently exploring the recently discovered ability of *Cc*PDH to convert L-guluronate to D-glucarate [27] to establish a large-scale production system for hexarates, which are bio-based platform chemicals.

Electron transfer pathway

Intramolecular (or inter-domain) electron transfer (IET) in CDH has been studied in much depth [28•], and similar IET occurs in *Cc*PDH, from the PQQ cofactor in the AA12 domain to the heme *b* in the AA8 domain. When two kinds of electron acceptors are used for measuring enzymatic activity, phenazinemethosulfate (PMS) reacts directly with the PQQ domain, whereas cytochrome *c* (cyt *c*) reacts via the AA8 domain of *Cc*PDH (Figure 4). Comparison of the pH-dependence of the activity between the isolated PQQ domain and the full length *Cc*PDH suggested that the IET reaction is the rate-limiting step and the pH-dependent process in the full length *Cc*PDH [29••]. Interestingly, since the AA8 can act as 'a built-in mediator' to shuttle electrons between the active site in the catalytic domain and an electrode, *Cc*PDH is capable of direct electron transfer (DET) to an electrode [21••]. Indeed, a *Cc*PDH-immobilized glassy carbon electrode shows catalytic currents during oxidation of L-fucose without the use of redox mediators [29••]. For the development of biosensors and biofuel cells, DET has important advantages compared to mediated electron transfer (MET), which needs a redox mediator [30]. Thus, *Cc*PDH is an excellent candidate for making DET-based bioelectronics devices as is CDH [31].

Biological functions

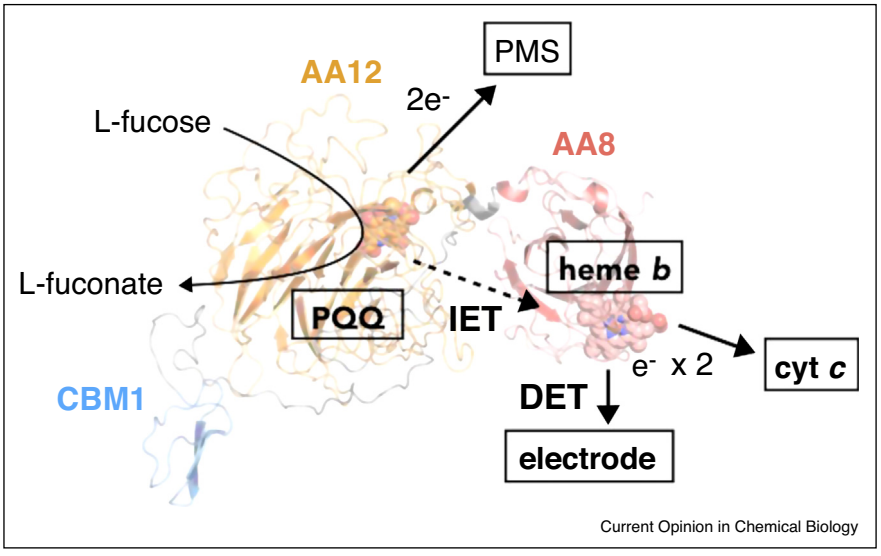
For *Cc*PDH, the occurrence of the non-catalytic, cellulose binding domain indicates a role in plant cell wall degradation, a complex process carried out by the synergistic action of hydrolases and oxidoreductases [17••].

Table 1

List of PQQ-dependent enzymes

Enzymes	Organisms	Major Substrates	Reference
6-bladed beta-propeller structure			
AA12 pyranose dehydrogenase	*Coprinopsis cinerea*	D-glucosone, L-fucose	[19••]
2-keto-D-glucose dehydrogenase	*Pseudomonas aureofaciens*	D-glucosone	[20•]
Soluble glucose dehydrogenase	*Acinetobacter calcoaceticus*	D-glucose	[42]
Aldose sugar dehydrogenase	*Escherichia coli*	various aldose sugars	[43]
	Thermus thermophilus		[44]
	Pyrobaculum aerophilum (archaea)		[45]
Sorbosone dehydrogenase	*Ketogulonicigenium vulgare*	L-sorbosone	[46]
8-bladed beta-propeller structure Soluble			
Methanol dehydrogenase	Methylotrophs	methanol	[47]
Alcohol dehydrogenase I	Proteobacteria	ethanol, propanol, butanol, polypropylene glycol	[13]
Alcohol dehydrogenase II	Proteobacteria	butanol, pentanol, propanediol, polyethylen glycol, tetrahydrofurfuryl alcohol	[13]
Lupanine hydroxylase	*Pseudomonas* species	lupanine	[48]
Polyvinylalcohol dehydrogenase	*Pseudomonas* sp., *Sphingomonas* sp.	polyvinylalcohol	[49]
8-bladed beta-propeller structure Membrane-bound			
Glucose dehydrogenase	Gram-negative bacteria	D-glucose	[50]
Glycerol dehydrogenase	*Gluconobacter* species	glycerol, sorbitol, arabitol, erythritol	[51]
Sorbitol dehydrogenase	*Gluconobacter suboxydans*	sorbitol	[52]
Quinate dehydrogenase	*Acinetobacter calcoaceticus*	quinate	[53]
	Gluconobacter oxydans		[54]
Alcohol dehydrogenase III	Acetic acid bactria	ethanol	[13]

Figure 4



Schematic presentation of possible electron transfer pathways in *Cc*PDH.
Electron pathways are shown as straight arrows. In the oxidative half reaction, a two electron acceptor such as phenazine methosulfate (PMS) is directly reduced by the reduced AA12 domain (PQQ), or a single electron acceptor such as cytochrome *c* (cyt *c*) is reduced via the heme *b* group in the AA8 domain. In the latter case, intramolecular electron transfer occurs from PQQ to heme *b*. Direct electron transfer (DET) occurs between the AA8 domain of *Cc*PDH and electrodes. The 3D structure model of *Cc*PDH was constructed using Protein Homology/analogY Recognition Engine (Phyre) version 2.0.

Interestingly, *Cc*PDH shows the highest oxidative activity toward 2KG, which is the product of glucose oxidation by pyranose-2-oxidase and pyranose dehydrogenase, which are produced by several fungi. It is thus conceivable that *Cc*PDH is a part of an alternative pathway in glucose metabolism. However, this hypothesis has yet to be confirmed and several observations argue against it: the affinity of *Cc*PDH for 2KG (and all the other substrates tested) is low and genes encoding 2KG-producing enzymes have not been identified in the genome of *C. cinerea*.

The interplay between the fungal PQQ-dependent dehydrogenase and LPMOs is of major interest. LPMOs are copper-dependent metalloenzymes that cleave polysaccharide chains oxidatively and act synergistically with hydrolases to degrade plant cell walls [17••,32,33,34•]. LPMO action requires exogenous electron donors, which may be enzymatic, such as CDH [35,36], or non-enzymatic, such as L-ascorbic acid [37] and various hydroquinones (e.g. diphenols) [38•]. It was recently shown that *Cc*PDH can activate two *Neurospora crassa* LPMOs (belonging to the AA9 family) and that the AA8 cytochrome domain is indispensable for such an activation to occur [25•]. Furthermore, removal of the CBM domain from *Cc*PDH has a negative impact on the efficiency of the *Cc*PDH-LPMO system. Since the cellulose binding CBM keeps *Cc*PDH associated with cellulose [21••], this observation suggests that electron transfer in the vicinity of the substrate is advantageous for the *Cc*PDH-LPMO system [25•]. The important roles of the AA8 domain and CBM1 may be taken to suggest that *Cc*PDH is tailored for activating LPMOs. It should be noted, however, that a considerable fraction of AA12 enzymes come without a CBM. Interestingly, 2KGA, the oxidation product of the most favorable *Cc*PDH substrate, 2KG, is a precursor of erythorbic acid, which is known as isoascorbic acid (i.e. a stereoisomer of ascorbic acid) [39]. It is thus conceivable that *Cc*PDH plays a role in the biosynthesis of L-ascorbic acid, which is a known good electron donor for LPMOs.

*Cc*PDH requires a PQQ molecule for its catalytic activity. It has been known that only a limited number of prokaryotic species can synthesize PQQ, while there is no report on PQQ synthesis in fungi or in other eukaryotes. Therefore, the supply route of PQQ to fungal AA12 enzymes is still unclear. It is well known that bacteria and fungi frequently co-occur in plant cell wall degradation and their symbiotic interactions have also been suggested [40]. Therefore, one plausible possibility is that the PQQ cofactor of the fungal quinoproteins is provided from the PQQ-producing bacteria. Of note, it has been predicted that PQQ plays various physiological roles in prokaryotic and eukaryotic organisms that do not synthesize PQQ [41]. The high affinity of *Cc*PDH to PQQ (K_d of 1.1 nM) indeed enables the enzyme to capture PQQ from the natural environment. However, at present, there is no clear evidence for a supply route of

PQQ to fungal AA12 enzymes, and it thus remains a mystery.

Conclusions

The PQQ-dependent *Cc*PDH is the first PQQ-dependent enzyme identified in eukaryotes. The PQQ cofactor binds the enzyme with high affinity, reflected in a K_d of 1.1 nM, and the enzyme oxidizes 2KG, L-fucose, and rare sugars such as D-arabinose and L-galactose. The AA8 cytochrome *b* domain exhibits spectral and electrochemical features that are almost identical to those observed for the AA8 domains of CDHs, suggesting that *Cc*PDH can activate LPMOs, as was indeed observed. The amino acid sequence of the AA12 domain in *Cc*PDH is clearly distinct from those of known PQQ-dependent enzymes, underpinning the novelty of this family of PQQ-dependent enzymes. The fungal PQQ-dependent enzymes provide an expanded perspective on the enzymatic degradation of plant polysaccharides, especially due to their ability to activate LPMOs. However, the physiological role of this enzyme remains unclear. Further studies on AA12 enzymes have a potential to generate new concepts in plant biomass degradation and may even shed light on symbiotic interplay between fungi and bacteria.

Conflict of interest statement

Nothing declared.

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