Crystal structures of *Phanerochaete chrysosporium* pyranose 2-oxidase suggest that the N-terminus acts as a propeptide that assists in homotetramer assembly

Noor Hassan\(^a\), Tien-Chye Tan\(^b\), Oliver Spadiut\(^a\), Ines Pisanelli\(^c\), Laura Fusco\(^c\), Dietmar Haltrich\(^c\), Clemens K. Peterbauer\(^c\), Christina Divne\(^a,b\,*

\(^a\)KTH Royal Institute of Technology, School of Biotechnology, Albanova University Center, Roslagstullsbacken 21, S-10691 Stockholm Sweden
\(^b\)Karolinska Institute, Department of Medical Biochemistry and Biophysics, Scheelelaboratoriet, Schees väg 2, S-17177 Stockholm Sweden
\(^c\)BOKU University of Natural Resources and Life Sciences, Food Biotechnology Laboratory, A-1190 Vienna Austria

**ABSTRACT**

The flavin-dependent homotetrameric enzyme pyranose 2-oxidase (P2O) is found mostly, but not exclusively, in lignocellulose-degrading fungi where it catalyzes the oxidation of \(\beta\)-D-glucose to the corresponding 2-keto sugar concomitantly with hydrogen peroxide formation during lignin solubilization. Here, we present crystal structures of P2O from the efficient lignocellulolytic basidiomycete *Phanerochaete chrysosporium*. Structures were determined of wild-type PcP2O from the natural fungal source, and two variants of recombinant full-length PcP2O, both in complex with the slow substrate 3-deoxy-3-fluoro-\(\beta\)-D-glucose. The active sites in PcP2O and P2O from *Trametes multisolor* (TmP2O) are highly conserved with identical substrate binding. Our structural analysis suggests that the 17 \(^\circ\)C higher melting temperature of PcP2O compared to TmP2O is due to an increased number of inter-subunit salt bridges. The structure of recombinant PcP2O expressed with its natural N-terminal sequence, including a proposed propeptide segment, reveals that the first five residues of the propeptide intercalate at the interface between A and B subunits to form stabilizing, mainly hydrophobic, interactions. In the structure of mature PcP2O purified from the natural source, the propeptide segment in subunit A has been replaced by a nearby loop in the B subunit. We propose that the propeptide in subunit A stabilizes the A/B interface of essential dimers in the homotetramer and that, upon maturation, it is replaced by the loop in the B subunit to form the mature subunit interface. This would imply that the propeptide segment of PcP2O acts as an intramolecular chaperone for oligomerization at the A/B interface of the essential dimer.

\(\odot\) 2013 The Authors. Published by Elsevier B.V. on behalf of Federation of European Biochemical Societies. Open access under CC BY-NC-ND license.

1. Introduction

The flavoprotein pyranose oxidase (P2O; EC 1.1.3.10) is found in most lignocellulolytic fungi [1–3] where it catalyzes the highly regioselective oxidation of D-glucose and generation of hydrogen peroxide as an integral part of the fungal degradative and metabolic machinery. Besides the general importance of P2O in wood decay and recycling, the enzyme is attracting interest based on its unusual mechanistic features and potential use in a wide range of applications, e.g., sugar biotransformation reactions, synthesis of rare sugars and fine chemicals [4], and in enzymatic biofuel cells [5].

P2O was first isolated from *Polyporus obtusus* [7], and later from additional wood-rotting fungi, including the highly efficient lignocellulose-degrading fungus *Phanerochaete chrysosporium* [9]. P2O from *P. chrysosporium* (PcP2O) is expressed together with other redox-active enzymes when lignin is used as carbon source and displays similar regulation patterns, which strongly implies that the principal function lies in lignin solubilization [10]. The 270-kDa large enzyme is homotetrameric with a subunit molecular mass of 68 kDa where each of the four identical chains carries one active site and binding pocket for flavin adenine dinucleotide (FAD) [12]. Like other P2Os, PcP2O catalyzes the oxidation of a number of aldopyranoses.

---

**Abbreviations:** 2FGl, 2-deoxy-2-fluoro-\(\beta\)-D-glucose; 3FGl, 3-deoxy-3-fluoro-\(\beta\)-D-glucose; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; IMAC, by immobilized metal ion affinity chromatography; IPTG, \(\beta\)-D-1-thiogalactopyranoside; MES, 2-(N-morpholino)ethanesulfonic acid; MWCO, molecular weight cut off; P2O, pyranose oxidase; PBS, phosphate buffered saline; PDB, Protein Data Bank; PEG, polyethylene glycol; TEV, Tobacco Etch Virus.

*Corresponding author at: KTH Royal Institute of Technology, School of Biotechnology, Albanova University Center, Roslagstullsbacken 21, S-10691 Stockholm Sweden.
Tel.: +46 8 55378296; fax: +46 8 55378468.
E-mail address: divne@biotech.kth.se (C. Divne).
preferably glucopyranose, at the C2 position yielding the corresponding 2-keto sugars [13].

The related Trametes multicorlor P20 (TmP20) has been shown to catalyze this reaction by a ping-pong type mechanism [14]. The overall reaction includes two half-reactions: a reductive half-reaction whereby β-α-GAC is oxidized at C2 to 2-keto β-α-Glc [14], the flavin adenosine dinucleotide (FAD) is reduced to FADH₂, and the keto sugar is released. The enzyme is highly regioselective catalyzing almost exclusively 2-oxidation of glucose [2,16–18]. Rare instances of C3 oxidation have been reported for some substrates [16]. The following oxidative half-reaction is pH-dependent [19], and involves the reduction of O₂ to H₂O₂ concomitantly with flavin re-oxidation. Oxygen activation during the oxidative half-reaction has been shown to involve a transient covalent flavin (4a)-hydroperoxide intermediate [19]. P20 from Peniophora gigantea was observed to oxidize β-δ-glucosidase at the C2 position, whereas 2-deoxy-β-glucopyranose was oxidized at the C3 position to yield 3-dehydroallose (3-ketoallose) [16]. This finding pinpointed an inherent ability of P20 to oxidize similar substrates at different positions, i.e., with different regioselectivity.

P20 has great potential in a variety of applications, including sugar biotransformation reactions, analytical tools, chemoenzymatic synthesis (reviewed in [4]), and biosensors or biofuel cells. 7. For the most common electron-donor substrates, 2- to 3-fold higher specificity constants (kcat / Km) have been observed for PcP20 compared with TmP20, and a similar trend is seen for common electron-acceptor substrates [21]. To be useful as a biocatalyst, an enzyme typically has to display considerable stability under operational conditions. In this context it is interesting to note that PcP20 is significantly more thermostable (Tm, 75.4 °C) than the homologs from Trametes multicolor and Lyophyllum shimeji [21], which display Tm values of 58.2 °C and 54.9 °C, respectively. Knowledge about the three-dimensional structure provides an important framework within which analysis of differences in thermostability can be discussed, and to suggest structural determinants to be targeted by mutagenesis to further fine tune catalytic and biophysical properties to meet the demands for industrial use in biocatalysis.

To date, crystal structures of P20 from only two fungal species have been reported, namely TmP20 [22] and a P20 from Peniophora sp. [23]. Whereas the Trametes and Peniophora P20s share 99.7% sequence identity with only two amino acids differing, the amino-acid sequence of PcP20 [10] is distinctly different, especially in the N-terminal region, with only 40% overall sequence identity to the other two P20s of known structure. To further elucidate how different P20s differ at the structural level, and how differences are manifested at functional and mechanistic levels, crystal structures of PcP20 from three different sources were resolved: the 1.80-Å structure of wild-type PcP20 from the natural source P. chrysosporium; the 1.80-Å structure of recombinant wild-type PcP20 (full-length gene coding for residues 1–621) expressed in Escherichia coli with a non-cleavable N-terminal T7-epitope tag; and the 2.40-Å structure of the PcP20 variant H158A (full-length gene coding for residues 1–621) expressed with a cleavable hexahistidine tag that was removed proteolytically to yield the full-length gene product with an authentic N-terminus. The latter two crystal structures were determined in the presence of the slow substrate 3-deoxy-3-fluoro-β-δ-glucosidase (3FGlC). Based on these structures, we compare PcP20 with other P20s of known structure with emphasis on thermal stability, and discuss the possibility of a propeptide functionality at the N-terminus.

2. Materials and methods

2.1. Production of wild-type PcP20s

Wild-type PcP20 from P. chrysosporium strain K-3 [24], referred to as PcP20NAWT, was produced in shake flask, and purified from mycelial extracts as previously described [3]. In brief, a combination of anion exchange and hydrophobic interaction chromatography was used to obtain an apparently homogenous enzyme preparation. The gene for PcP20 (UNP Q6QRW1) coding for the 621 amino acid subunit of PcP20 has been previously cloned into the bacterial expression vector pET21a (+) carrying an N-terminal T7-epitope tag (coding for the amino-acid sequence 1-24)EMSMTGGQMQGRGS-1) where 2-RGS are additional cloning artifacts introduced by the restriction site, as well as a non-cleavable C-terminal hexahistidine tag (amino-acid sequence 24-23-KAAALEHHHHHHH34), and expressed in E. coli strain BL21 (DE3), and produced as reported earlier [12]. The recombinant wild-type variant is referred to as PcP20RECWT.

2.2. Cloning, expression and purification of PcP20 variant H158A (PcP20RECWT)

To generate the PcP20 mutant H158A, the P. chrysosporium p2a gene from the original vector [12] was subjected to site-directed mutagenesis using PCR. The template, pLFO7, and the primers PcP20-H158A_fw (5’-GCATGAGGACTGCCTGACGTGGCCAC-3’); PcP20-H158A_rev (5’-GCTATGGCCGCCCAGGCGTCGAC-3’) were used for mutagenic PCR reactions. The PCR reaction contained 2.5 μl Pfu DNA polymerase (Fermentas, Germany), 100 ng of plasmid DNA, 5 pmol of each primer, 10 μM of each dNTP and 1× PCR buffer (Fermentas) in a total volume of 50 μl. The following conditions were used for mutagenic PCRs: 95 °C for 4 min, then 30 cycles of 94 °C for 30 s; 65 °C for 30 s; 72 °C for 16 min, with a final incubation at 72 °C for 10 min. Following PCR, the methylated template-DNA was degraded by digestion with 10 U of DpnI at 37 °C for 3 h. The remaining PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega, USA). PCR products (5 μl) were transformed into electro-competent E. coli BL21 Star DE3 cells. To prove that only the desired mutation had occurred, the mutated PcP20-encoding gene was sequenced by a commercial provider (VBC Biotech, Vienna, Austria) using primers T7prom_f (5’-AAATAGACACTGATATAG-3’) and T7term_rev (5’-GCTATGTTATGTCGTACGGC-3’).

The gene was amplified by standard PCR, and the insert cloned into the pNIC28-Bsa4 vector [25] using ligation-independent cloning [26]. The vector adds 23 residues comprising a hexahistidine (His6) tag and a Tobacco Etch virus (TEV) protease cleavage site to the N-terminus of the expressed protein (sequence 2-349HHHHHHSSVGLTENLYFQM-35). Cleavage with TEV protease leaves the sequence 2-348-35 the N-terminus. To reduce the number of additional N-terminal residues, the naturally occurring N-terminal methionine in the pcP20 gene was replaced by the translation start Met, leaving only an additional serine. The recombinant plasmid expressing His6-TEV-PcP20 was initially transformed into the E. coli cloning strain MachI (Invitrogen) grown on Luria Bertani (LB) agar plates supplemented with 5% sucrose and 50 μg/mL kanamycin for the selection of recombinant plasmids with cleaved SacB (levansucrase).

Recombinant plasmids were isolated from MachI cells using plasmid preparation with the QiAprep Spin Miniprep Kit (Qiagen), followed by plasmid transformation into the E. coli expression strain BL21(DE3). Transformed BL21(DE3) cells were grown in 0.6 L Terrific Broth (TB) medium supplemented with 50 mg/mL kanamycin, 60 mL glycerol (per 600 mL), 4 mM glucose, and 0.8 mM MgSO₄. The cultivation broth was inoculated with 7 mL overnight seed culture of BL21(DE3) and allowed to grow at 37 °C with constant shaking at 200 rpm until reaching an OD₆₀₀ of 0.7, at which PcP20 expression was induced with 0.24 mM isopropyl β-1-thiogalactopyranoside (IPTG). The induced culture was grown for 18 h at 18 °C.

Cells were harvested by centrifugation at 4 °C (8983 rcf) using Avanti J-170XP centrifuge (Beckman) with rotor JLA 8.1000 for 15 min. The bacterial cell pellet was resuspended in 3 volumes of lysis buffer
(phosphate buffered saline, PBS). The sample was homogenized using AVESTIN Emulsiflex-C3, and the lysate collected in a beaker on ice. The lysate was then centrifuged at 4 °C, (3900 ref) using Avanti J-20 XP centrifuge (Beckman) with rotor JA 25.50 for 30 min to pellet the cell debris.

A 2-ml Ni²⁺-charged immobilized metal affinity chromatography (IMAC) Ni-NTA agarose resin (Invitrogen) was washed and equilibrated with lysis buffer. The clear lysate was passed through the column and the flow through collected, followed by 2 column volumes (CVs) of washing with wash buffer [PBS, 20 mM imidazole] to elute non-specifically binding proteins. Pcp20 was eluted with elution buffer [PBS, 500 mM imidazole]. To remove the His6 tag, the protein was incubated at 1:100 ratio with TEV protease in a dialysis bag (molecular weight cut off, MWCO, 12–14 kDa) containing 50 mM Tris, 0.5 mM EDTA and 1 mM diethiothreitol (pH 8.0), and left at 4 °C overnight. Following TEV protease treatment, Pcp20 was subjected to a second round of Ni²⁺-IMAC purification, and the flow through containing the untagged protein was collected.

The protein sample was concentrated to 35 mg/mL using a Vivaspin® centrifugal concentrator (MWCO 50 kDa), and further purified by size-exclusion chromatography using a HiLoad™ 16/60 Superdex™ 200 prep grade column (GE Healthcare Life Sciences) equilibrated with 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) pH 7.2, and 150 mM NaCl. Purity of the Pcp20 preparation was assessed by SDS–PAGE. Protein fractions were pooled and concentrated to 12 mg/mL using the Vivaspin® centrifugal concentrator (MWCO 50 kDa), and used as stock solution for subsequent crystallization screening and optimization plates.

### 2.3. Crystallization and data collection

The vapor diffusion hanging drop method was used for all Pcp20 crystallization experiments, and screening performed using commercially available screens: PACT suite (Qiagen), JCSG + suite (Qiagen), and Crystal Screen HT (Hampton Research). Pcp20_NATWT crystals were of space group P2₁2₁2₁ with one homotetramer in the asymmetric unit (asu). The crystals grew from hanging drops mixed by equal volumes of 15 OD₂₈₀/mL protein stock and reservoir containing 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES)-OH buffer (pH 5.2), 0.2 M MgCl₂, 32% (w/v) polyethylene glycol (PEG) 400. Pcp20_RECWT crystals were of space group P2₁ (one homotetramer per asu) and grew from drops prepared by equal volumes of 22 mg/mL protein [in 50 mM HEPES (pH 6.5), 150 mM NaCl] and reservoir [containing 0.1 M Tris–HCl (pH 8.5), 0.1 M MnCl₂, 30% (w/v) PEG 400]. Pcp20_RECWT/SA crystals belonging to space group P2₁ (two homotetramers per asu) were crystallized from equal volumes of a protein stock (13 mg/mL Pcp20 in 0.2 M HEPES, pH 7.2) and reservoir solution containing 0.2 M NH₄Cl, 15% (w/v) PEG 6000, and 0.1 M HEPES (pH 7.0). All crystals were yellow in color indicating unmodified oxidized enzymes.

The ligand complexes were prepared as followed: the Pcp20_RECWT crystal was immersed in a reservoir solution containing 3FGlc; and the Pcp20_RECWT/SA crystal was briefly exposed to a saturated solution of 3FGlc dissolved in 0.2 M NH₄Cl, 25% (w/v) PEG 6000, 0.1 M HEPES (pH 7.0). All crystals were vitrified in liquid nitrogen prior to data collection. Intensity data were collected at 100 K using synchrotron radiation at beamline I91-2 and I91-3, MAX-lab (Lund, Sweden), followed by data processing and scaling using the XDS package [27]. See Table 2 for crystal and data collection statistics.

### 2.4. Structure determination and refinement

Phasing was performed by molecular replacement using Phaser [28] included in the PHENIX suite [29] using the refined model of Tmp20 variant H167A as starting model (PDB: 3PL8 [18]). For Pcp20_NATWT and Pcp20_RECWT crystallographic refinement was performed using PHENIX initially, and with REFMAC5 [30] during the later stages of refinement. Refinement included anisotropic scaling, calculated hydrogen scattering from riding hydrogens, and atomic displacement parameter refinement using the translation, libration, screw–rotation (TLS) model. TLS models were built using the TLS Motion Determination server [32]. Pcp20_RECWT was refined with PHENIX throughout. Individual isotropic temperature factors were refined, and TLS was included in the last refinement round. All model building was done manually using the program O [33] and Coot [34] guided by σₐ-weighted 2Fo − Fo and Fo − Fo electron-density maps. The same sets of Rfree reflection were used throughout refinement. Missing loop residues due to flexibility and disorder were not modeled, and include for Pcp20_NATWT, A₁–A₁₂, A₅₇–A₆₄, A₃₁₁–A₃₁₈, A₃₄₉–A₃₆₅, A₆₁₈–A₆₂₀, B₁–B₁₂, B₃₁₁–B₃₁₈, B₃₅₀–B₃₆₅, B₆₁₈–B₆₂₀, C₁–C₁₂, C₅₇–C₆₄, C₃₁₁–C₃₁₈, C₃₄₉–C₃₆₅, C₆₁₈–C₆₂₀, D₁–D₁₂, D₅₇–D₆₄, D₃₁₁–D₃₁₈, D₃₄₉–D₃₆₅, D₆₁₈–D₆₂₀; for Pcp20_RECWT, A₁–A₁₂, A₅₇–A₆₅, A₃₁₀–A₃₁₈, A₃₄₉–A₃₆₅, A₆₁₈–A₆₂₀, B₁–B₉, B₅₈–B₆₅, B₃₁₀–B₃₁₈, B₃₄₉–B₃₆₅, B₆₁₈–B₆₂₀, C₁–C₁₂, C₃₁₀–C₃₁₈, C₃₄₉–C₃₆₅, C₆₁₈–C₆₂₀, D₁–D₁₂, D₅₇–D₆₅, D₃₁₀–D₃₁₈, D₃₄₉–D₃₆₅, D₆₁₈–D₆₂₀; and for Pcp20_RECWT/SA, 57–69, 31₀–31₈ and 6₁₈–6₂₀ for all eight monomers A–H in two homotramers. In addition, the following loop residues are missing: A₃₄₉–A₃₆₇, B₃₄₉–B₃₆₅, C₃₄₉–C₃₆₅, D₃₄₉–D₃₆₅, E₃₄₉–E₃₆₇, F₃₄₉–F₃₆₅, C₃₄₉–C₃₆₅ and H₃₄₉–H₃₆₅. Model refinement statistics are given in Table 3. All pictures showing structures were made with the program PyMOL [35].

### Table 1

Details of interactions between F2Os and 3-fluorinated glucose.

| Active-site conformer | productive 2-oxidation binding mode | productive 2-oxidation binding mode |
|-----------------------|--------------------------------------|--------------------------------------|
|                       | Tmp20RECWT                           | Pcp20RECWT                           |
| Sugar–protein interactions | β-3FGLc | β-3FGLc |
| O1                    | A546 O₁                               | A551 O₁                              |
| O2                    | H448 N₂                               | H553 N₂                              |
| O2                    | N593 N₂                               | N596 N₂                              |
| F3                    | Q448 N₂                               | Q454 N₂                              |
| F4                    | D452 O₅                               | D458 O₅                              |
| O6                    | Y456 O₁                               | Y462 O₁                              |

a PDB code 3PL8.

b The following three criteria are considered consistent with a productive binding mode: (i) the sugar is oriented for oxidation at C2; (ii) the substrate-binding loop is in the semi-open conformation; (iii) the side chain O₁ of Thr169 is pointing away from the flavin N(5)/O(4) locus.

c Italized interactions represent interactions with the catalytic residues.
Table 2

| Protein variant | PcP2O\textsubscript{NATWT} | PcP2O\textsubscript{RECVGT} / 3FGic | PcP2O\textsubscript{RECIVSA} / 3FGic |
|-----------------|---------------------------|---------------------------------|---------------------------------|
| Source/Vector   | Natural source, P. chrysosporium | Recombinant, E. coli/pET21a (+) | Recombinant, E. coli/pNIC28-Bsa4 |
| Cell constants  | a: 164.0, 164.0, 232.5 | P2\textsubscript{1}, 21/4 | P2\textsubscript{1}, 8 |
| Space group / mol. per asu | P3\textsubscript{1}, 21/4 | P2\textsubscript{1}, 21/4 | P2\textsubscript{1}, 8 |
| Beamline, λ (Å) | Max-lab, IJ11-3, 1.0000 | Max-lab, IJ11-3, 1.0000 | Max-lab, IJ11-2, 1.04094 |
| Resolution range, (Å) | 47.43 – 1.80 | 46.95 – 1.80 | 48.56 – 2.40 |
| nominal (Å) | (1.90 – 1.80) | (1.90 – 1.80) | (2.50 – 2.40) |
| Unique reflections | 331, 514 (49, 157) | 235, 360 (35, 011) | 233, 009 (26, 556) |
| Multiplicity | 11.1 (11.2) | 3.8 (3.8) | 4.9 (4.8) |
| Completeness (%) | 99.9 (99.9) | 99.0 (98.6) | 98.4 (97.4) |
| I/σ(I) | 17.1 (2.1) | 12.8 (2.3) | 11.9 (1.7) |
| R\textsubscript{mono} (I) (σ(I)) | 11.9 (200.3) | 11.8 (102.8) | 10.0 (138.3) |
| CC(1/2) | 95.9 (76.3) | 95.6 (38.8) | 95.8 (62.6) |

\* The outer shell statistics of the reflections are given in parentheses. Shells were selected as defined in XDS [27] by the user.
\* R\textsubscript{mono} = \|F\textsubscript{o} – |F\textsubscript{i}|\|/\|F\textsubscript{o}\| \times 100%.
\* CC(1/2) = Percentage of correlation between intensities from random half-data sets. Values given represent correlations significant at the 0.1% level [51].

Table 3

| PDB accession code | 4MIF | 4MIG | 4MIH |
|-------------------|------|------|------|

3. Results

3.1. Overall structure of PcP2O

The overall structure of PcP2O is very similar to that of TmP2O (PDB: 1TT0 [22]). A structure-based amino-acid sequence alignment highlights the most important differences (Fig. 1). The main differences include five insertions in TmP2O (boxed regions A–E in Fig. 1), and one major deletion (boxed region F in Fig. 1). The homotrimer is built up from a dimer of dimers where the A–B and C–D subunit pairs form two essential dimers, respectively, each with an extensive intersubunit interface (Fig. 2A). Insertion A in the sequence alignment is a 22-residue segment (residues 51–72) that corresponds to a loop (Fig. 2B) participating in oligomerization by providing intersubunit interactions with the nearby B subunit. This loop is ordered in only one subunit of the two wild-type PcP2O structures, namely subunit B in PcP2O\textsubscript{NATWT}, and subunit C in PcP2O\textsubscript{RECVGT}. In other subunits of PcP2O homotramers there is no interpretable electron density for residues 57–65 in this oligomerization loop. Insertion B comprises eight residues (residues 169–176) of a surface loop with no apparent role in subunit interactions. Insertion C (residues 230–232) provides an additional turn in an α-helix in the substrate-binding domain. Insertion D (residues 310–317) and E (residues 353–367) constitute surface-exposed loops that lack interpretable electron density in all subunits, and are therefore assumed to be highly flexible. The region in TmP2O that is deleted in PcP2O (Fig. 1) comprises residues Thr381 to Thr407 (TmP2O numbering), which is part of the “head” domain in TmP2O (Fig. 2B). The function of this domain is unknown but has been noted to have a remarkably flat surface and be rich in threonine and serine residues [22]. The truncation of the head domain in PcP2O effectively removes the β-hairpin that constitutes the flat surface in TmP2O (Fig. 2B).

3.2. Binding of 3-fluorinated glucose

Ligand complexes have been reported previously for TmP2O [18,36] and Peniophora P2O (PDB: 2FSV; [23]). In the case of Peniophora P2O, the authors claimed to have captured a complex with the product, 2-keto-β-glucose. However, at closer inspection of the experimental data, the electron density in the active site was better explained by composite binding of two partly occupied binding modes of glucose, i.e., Glc oriented for oxidative deamination at C2, and the competing binding mode where Glc is oriented for oxidation at C3 [18]. Ordered high-resolution complexes of these two principal glucose-binding modes have been described in detail for TmP2O (C2-oxidation mode, PDB: 3PL8 [18]; C3-oxidation mode, PDB: 2IG0 [36]), and we therefore have limited the structural comparison presented here to TmP2O.

The active sites of PcP2O and TmP2O are highly conserved. Recombinant wild-type PcP2O (Fig. 3A) and H158A PcP2O (Fig. 3B) bind 3FGic in the productive 2-oxidation binding mode as observed for the H167A variant of TmP2O (PDB: 3PL8 [18]). The ligand binds identically in all three complexes TmP2O–3FGic, PcP2O\textsubscript{RECVGT}–3FGic and PcP2O\textsubscript{H158A}–3FGic (Fig. 3C). Details of protein-ligand interactions are given in Table 1. As defined for the productive 2-oxidation binding mode of β-glucose [18], the substrate-recognition loop is in the semi-open conformation, and the mechanistically important Thr160 (corresponding to Thr169 in TmP2O) points away from the flavin N(5)/O(4) locus.

The only notable difference between the three structures is that...
Thr160 in PcP2O<sub>RECWT</sub> shows subtle signs of a mixture of two alternative rotamer conformations: i.e., the rotamer observed for the occluded P2O state where the threonine side-chain hydroxyl group hydrogen bonds to the flavin N(5)/O(4) [23]; and the rotamer that points away from the flavin as observed in substrate-bound active-site conformers relevant to the reductive half-reaction [18,36]. This rotamer ambiguity is not present in the redox-suppressed variants of P2O<sub>RECWT</sub> (variant H158A) and TmpP2O (variant H167A). The rotamer ambiguity in P<sub>cP2O<sub>RECWT</sub></sub> is probably due to the higher rate of flavin reduction than in the flavinylating-ligand mutants. Reduced flavin reduction has been shown for the H167A variant of TmpP2O [17]. Due to the high catalytic efficiency of recombinant wild-type TmpP2O it proved necessary to use the H167A variant in order to obtain crystal complexes with ordered bound 3- and 2-fluorinated glucose [18,36]. Binding of both 2- and 3-fluorinated glucose has been observed for wild-type TmpP2O but with a certain degree of disorder resulting in partly occupied states (unpublished results). Importantly, the structure of P<sub>cP2O<sub>RECWT</sub></sub> complex conclusively proves that the binding observed for 3FGlc in the redox-suppressed mutants of TmpP2O and P<sub>cP2O</sub> is identical and not introduced by the mutation. The reason for the more ordered binding of 3FGlc to wild-type P<sub>cP2O</sub> compared to TmpP2O is unknown at this point, but may be due to an overall lower flavin-reduction rate of 3FGlc by wild-type P<sub>cP2O</sub>.

The only sequence-related differences near the glucose-binding site are Met388 and Ala551 in P<sub>cP2O</sub>, which correspond to Leu361 and Val546 in TmpP2O. Despite these replacements, 3FGlc assumes an identical conformation, position and protein interactions as in TmpP2O. In P<sub>cP2O</sub> from the natural source without bound substrate, the substrate-recognition loop is in the fully open conformation with Thr160 pointing away from the flavin N(5)/O(4) locus. This conformation represents the unliganded state of P2O, and is most similar to that of the open conformer of TmpP2O H167A in complex with 2-fluorinated glucose [36].
3.3. Interactions provided by the N-terminus in the different PcP2O variants

The PcP2O homotetramer is a dimer of dimers where A/B subunits and C/D subunits form essential dimers with extensive dimer interaction surfaces. The dimers are further assembled into homotetramers, mainly through interactions by the long “oligomerization arms” comprising residues 116–143 in TmpP2O [22], and residues 105–134 in PcP2O. According to UniProtKB, the first 9 residues (MFLDTTPFR) of the PcP2O amino-acid sequence (UniProtKB Q6QWR1) are annotated as a propeptide sequence (P0000012348). We expressed recombinant wild-type PcP2O and H158A PcP2O in E. coli (PcP2ORECWT and PcP2ORECH158A) including the putative propeptide in the gene constructs. PcP2ORECWT was cloned into the pET21a (+) vector retaining an additional non-cleavable T7-tag at the N-terminus (before the suggested propeptide sequence), whereas the PcP2ORECH158A variant was cloned into the LIC vector pNIC28-Bsa4 with an N-terminal hexahistidine tag that was subsequently removed by Tobacco Etch Virus (TEV) protease to yield the more authentic N-terminal sequence SFLDTTPFR where the (−1) serine residue is left from the TEV-protease recognition sequence. The precise starting point of the polypeptide chain for PcP2O purified from the natural source is unknown due to the possibility of post-translational modification of the N-terminus such as possible proteolytic maturation cleavage. However, all three PcP2O constructs crystallized in different space groups, which is likely to reflect differences in the primary structure, also for PcP2ONATWT.

In the crystal structure of PcP2O from the fungal source (PcP2ONATWT), Pro13 is identified as the first residue at the N-terminus of all four subunits of the homotetramer. There is no visible electron density before Pro13 in any of the subunits. This does not mean that the naturally produced enzyme starts at Pro13, only that there is no interpretable electron density for residues 1–12 at the N-terminus. This can be due to removal of the N-terminus (maturation) or high flexibility. In the crystal structure of wild-type PcP2ORECWT, the N-terminally positioned T7-tag is forced into the surrounding solvent and not visible in the electron-density map, the first visible residue being Ala10 in subunit B, and Pro13 in subunits A, C and D. The possibility of proteolytic trimming of the N-terminus cannot be excluded, but in this case flexibility due to the N-terminally placed tag is likely.

In the crystal structure of PcP2ORECH158A, which has a more authentic N-terminus including the putative propeptide sequence without a leading tag (carrying only an additional serine at the N-terminus), the N-terminus is wedged between the A and B subunits (or C and D subunits) to provide inter-subunit interactions at the A/B (or C/D) dimer interfaces of the essential dimer of the 222 tetramer. Residues
The considera-

tion of the 

one-dimensional 

electron density. 

4. Discussion

Despite the low sequence identity of 40%, the overall structure of Pcp20 is similar to that of Tmp20. In particular, the active site is structurally highly conserved, which is clearly reflected in similar kinetic behavior [12]. The regioselectivity of C2 versus C3 oxidation has been studied in detail for wild-type and mutant Tmp20s with natural and fluorinated substrates using crystal-structure analysis [16,18] and biochemical approaches [14,17]. Binding modes for D-glucose oxidation at C2 [18] and C3 [36] are related by a simple 2-fold rotation of glucose about an axis defined roughly by a line running through a point midway between the atoms C5/O5 and a point midway between atoms C2 and C3. The two orientations in Tmp20 are subtly different with nearly identical sets of protein–substrate interactions, however, with two key side-chain interactions (O4–Asp452 O62 and O6–Tyr456 On) in favor of the 2-oxidation mode [18]. The active-site residues are conserved in Pcp20, including the catalytic His553–Asn596 pair (His548 and Asn593 in Tmp20), and the dynamic substrate-recognition loop (residues 457–467 in Pcp20; 451–461 in Tmp20).

As shown here by the two crystal structure complexes of recombinant Pcp20 (wild-type and H158A) with 3FGlc, the productive binding mode for 2-oxidation of D-glucose is identical to that previously established for Tmp20 [18]. Furthermore, the fact that both recombinant wild-type and flavinylation mutant (H158A) bind 3FGlc identically, proves that the flavinylation per se does not significantly alter the structural details of substrate binding. In the case of wild-type Tmp20, 3FGlc was turned over at an appreciable rate leading to disorder of the ligand in the active site, making it necessary to use a flavinylation mutant (Tmp20 H167A) to capture well ordered substrate-binding modes [18,36]. The ordered ligand structure of recombinant wild-type Pcp20 with 3FGlc presented here indicates that turnover might be considerably slower in this case.

In light of the high structural similarity between Tmp20 and Pcp20, the difference in melting temperature (17 °C higher for Pcp20) is surprisingly large [21]. The increased thermal stability of Pcp20 is likely to be attributed to a large number of subtle differences at the structural level that may not be easily deconvoluted. The general importance of ionic interaction networks for thermal stability of proteins has been recognized and discussed [37–42]. To investigate the possible influence of salt bridges on the different stability of Pcp20 and Tmp20, a comparison was made using ESBL analysis [43]. The result was surprising in that—although Tmp20 has a higher total number of salt bridges compared with Pcp20—the ionic links between subunits of the Tmp20 homotetramer are very few (PDB: 1T70 [22]). Only four intersubunit ion links are present out of a total of 247 possible salt bridges (two are formed across the A/B interface, and two across the C/D interface), the rest are intrasubunit ionic interactions. In Pcp20, however, the total number of possible salt bridges is 195, of which 20 are formed between individual subunits across the subunit interfaces (six across the A/C interface, four across the A/D interface, six across the B/D interface, and four across the B/C interface). Thus, the principal determinant likely to contribute to increased thermal stability of Pcp20 is the increased number of ionic links formed between subunits of the homotetramer rather than within the subunits.

There is very limited understanding of the functional and structural properties of peptidases, and it is therefore of particular interest to address this question in more detail. Most studies have been concerned with the role of prosegments in the activation of proteases [44]. In the case of redox-active enzymes, one example of interest is lignin peroxidase (LIP; UNP P06181) from P. chrysosporium. The enzyme is exported from the hypha via the secretory pathway, and contains a 21-residue long N-terminal leader sequence followed by a 7-residue propeptide (PRO_00000023760 [45]). The signal peptide is cleaved after Ala21 [46], and the propeptide is removed by cleavage at a dibasic site after Arg28 [46]. The role of the propeptide in
LIP is not known. Propeptide sequences typically show very low, if any, sequence similarity, and their precise role in folding or activation also differs between different proteins. The possible existence of a propeptide sequence has also been discussed for P2Os. This has been based on the highly heterogeneous N-termini experimentally characterized for various P2Os, although conclusive evidence is still missing [3, 15, 48].

Based on analysis with SignalP [SignalP 4.1: http://www.cbs.dtu.dk/services/SignalP [50]], the N-terminal sequence of PcP2O is not predicted as a secretion signal, which is expected since P2O is an intracellular protein. Previously observed extracellular release of P2O is most likely through autolysis [10]. Interestingly, it has been reported that the N-terminus of naturally produced PcP2O is susceptible to proteolytic degradation when subjected to cycles of freeze-thawing, and analysis of the proteolytically modified protein by means of LC–MS/MS identified Ala10 as the first amino acid present at the N-terminus [10]. This would be consistent with our PcP2O_{NATWT} structure where the electronic density for the N-terminus is visible from Pro13 (due to lack of crystal contacts, 10ADE [12] may be flexible). In PcP2O_{RECWT}, one subunit indeed shows density from N1 to Ala10. Whether the propeptide chain has been cleaved between Arg9 and Ala10 to eliminate the T7-epitope tag and the following propeptide segment, or whether the tagged N-terminus simply resides flexible in the solvent region is unknown. Regardless, it is evident that in PcP2O_{NATWT} and PcP2O_{RECWT}, the N-terminal segment is not placed at the A/B subunit interface where it is found in PcP2O_{REC/5S}. In both wild-type structures, the 66QFG68 loop of the neighboring subunit has replaced the N-terminus, which may correspond to the subunit interface architecture of mature PcP2O.

In the case of PcP2O_{REC/5S}, a vector and gene construct was used that enable the expression of a more authentic N-terminus. In this construct the entire propeptide is present with an additional serine residue preceding the N-terminal methionine residue. The extra serine is a remnant from the TEV protease recognition sequence and is therefore not possible to eliminate. In the PcP2O_{REC/5S} structure, the N-terminus is favorably wedged at the interface between A and B subunits, placing the N-terminal methionine from each subunit on each side of the 2-fold axis of the essential A/B dimer. The interactions formed by the propeptide in subunit A with the segment defined by residues 50–53 in subunit B of PcP2O_{REC/5S} are replaced by interactions offered by the 66QFG68 loop in subunit B in PcP2O_{NATWT} and PcP2O_{RECWT}. This may indicate that the N-terminal sequence serves as a transient propeptide that nucleates essential interactions between A and B subunits of the essential dimer (or C/D dimers) in the 222 tetramer. Thus, the N-terminal segment may serve the function of an intramolecular chaperone that stabilizes the A/B interface prior to sequestering the 66QFG68 loop of subunit B to assume its final position and conformation at the A/B interface of the mature enzyme. The propeptide segment does not significantly alter the dimer or homotrimer assembly, suggesting that its ability to pack favorably at the interface is an evolved feature, rather than an artifact. Although the present crystal structures cannot serve as absolute proof for the existence of a propeptide chaperone in PcP2O with a role in folding and/or oligomerization, our results and hypothesis provide a basis for further investigations regarding the putative existence and role of propeptides in oligomeric proteins. Site-directed mutagenesis experiments may provide useful, however, caution must be taken to carefully design variants since any amino-acid replacement or deletion may, directly or indirectly, artificially stabilize or destabilize the subunit interface, or alter the folding/oligomerization pathway, rather than to provide information about inherent intramolecular chaperon activity of the N-terminus in folding and/or oligomerization.

5. PDB accession numbers

Coordinates and structure factors are available in the Protein Data Bank database under the following accession numbers: wild-type PcP2O from natural source, PDB: 4MIF; recombinant wild-type PcP2O with 3FGlc, PDB: 4MIG; and PcP2O H158A with 3FGlc, PDB: 4MIH.

Acknowledgements

The authors thank the beamline staff at the MAX II beamlines I911–2 and I911–3 (MAX-LAB, Lund, Sweden) for assistance during data collection. C.D. and T.-C.T. were supported by grants from the Swedish Research Council VR (VR grants 2008–4045 and 2011-5768), The Austrian Science Fund is acknowledged for support to C.K.P. (FWF grants L210 and P22094) and O.S. (Erwin-Schrödinger Fellowship).

References

[1] Daniel, G., Volc, J., Kubáňová, E. and Nilsson, T. (1992) Ultrastructural and immunocytochemical studies on the H2O2-producing enzyme pyranose oxidase in Phanerochaete chrysosporium grown under liquid culture conditions. Appl. Environ. Microbiol. 58, 3667–3676.
[2] Daniel, G., Volc, J. and Kubáňová, E. (1994) Pyranose oxidase, a major source of H2O2 during wood degradation by Phanerochaete chrysosporium, Trametes versicolor, and Oudemansiella polymorpha. Appl. Environ. Microbiol. 60, 2524–2532.
[3] Artolozaga, M.J., Kubáňová, E., Volc, J. and Kalisz, H.M. (1997) Pyranose 2-oxidase from Phanerochaete chrysosporium – further biochemical characterisation. Appl. Microbiol. Biotechnol. 47, 508–514.
[4] Giffhorn, F. (2006) Fungal pyranose oxidases: occurrence, properties and biotechnological applications in carbohydrate chemistry. Appl. Microbiol. Biotechnol. 74, 727–740.
[5] Tasca, F., Timur, S., Ludwig, R., Haltrich, D., Volc, J., Antochia, R. et al. (2007) Amperometric biosensors for detection of sugars based on the electrical wiring of different pyranose oxidases and pyranose dehydrogenases with osmium redox polymer on graphite electrodes. Electroanalysis 19, 294–302.
[6] Tamaki, T., Ito, T. and Yamaguchi, T. (2007) Immobilization of hydroquinone through a spacer to polymer grafted on carbon black for a high-surface-area biofuel cell electrode. J. Phys. Chem. B 111, 10312–10319.
[7] Janssen, F.W. and Ruelius, H.W. (1968) Carbohydrate oxidase, novel enzyme from Polyporus obtusus. II. Specificity and characterization of reaction products. Biochim. Biophys. Acta 167, 501–510.
[8] Ruelius, H.W., Kerwin, R.M. and Janssen, F.W. (1968) Carbohydrate oxidase, a novel enzyme from Polyporus obtusus. I. Isolation and purification. Biochim. Biophys. Acta 167, 493–500.
[9] Volc, J. and Eriksson, K.-E. (1988) Pyranose 2-oxidase from Phanerochaete chrysosporium. Methods Enzymol. 161, 316–322.
[10] De Koker, T.H., Mozuch, M.D., Cullen, D., Gaskell, J. and Kersten, P.J. (2004) Isolation and purification of pyranose 2-oxidase from Phanerochaete chrysosporium and characterization of gene structure and regulation. Appl. Environ. Microbiol. 70, 5794–5800.
[11] Manavalan, A., Adav, S.S. and Sze, S.K. (2011) iTRAQ-based quantitative secreto- tome analysis of Phanerochaete chrysosporium. J. Proteome. 75, 642–654.
[12] Picaniello, L., Kujawa, M., Spaduti, O., Kitt, R., Halada, P., Volc, J. et al. (2009) Pyranose 2-oxidase from Phanerochaete chrysosporium – expression in E. coli and biochemical characterization. J. Biotechnol. 142, 97–106.
[13] Volc, J., Kubatova, E., Daniel, G. and Prikrylova, V. (1996) Only C2-specific glucose oxidase activity is expressed in ligninolytic cultures of the white rot fungus Phanerochaete chrysosporium. Arch. Microbiol. 165, 421–424.
[14] Prongjit, M., Sucharitkul, J., Wongnate, T., Haltrich, D. and Chaiyen, P. (2009) Ki- netic mechanism of pyranose 2-oxidase from Trametes multicolor. Biochemistry 48, 4170–4180.
[15] Leitner, C., Volc, J. and Haltrich, D. (2001) Purification and characterization of pyranose oxidase from the white rot fungus Trametes multicolor. Appl. Environ. Microbiol. 67, 3630–3637.
[16] Freimund, S., Huwig, A., Giffhorn, F. and Köpper, S. (1998) Rare keta-aldooses from enzymatic oxidation: substrates and oxidation products of pyranose 2-oxidase. Chem. Eur. J. 4, 2442–2455.
[17] Sucharitkul, J., Wongnate, T. and Chaiyen, P. (2010) Kinetic isotope effects on the non-covalent flavin mutant protein of pyranose 2-oxidase reveal insights into the flavin reduction mechanism. Biochemistry 49, 3753–3765.
[18] Tan, T.C., Haltrich, D. and Divine, C. (2011) Regioselective control of β-D-glucose oxidation by pyranose 2-oxidase is intimately coupled to conformational degen- eracy. J. Mol. Biol. 409, 588–600.
[19] Sucharitkul, J., Prongjit, M., Haltrich, D. and Chaiyen, P. (2008) Detection of a C4α-hydroperoxys flavin intermediate in the reaction of a flavoprotein oxidase. Biochemistry 47, 8490–8495.
[20] Prongjit, M., Sucharitkul, J., Palley, B.A. and Chaiyen, P. (2013) Oxidation mode of pyranose 2-oxidase is controlled by pH. Biochemistry 52, 1437–1445.
[21] Salaheddin, C., Takakura, Y., Tsunashima, M., Stranzinger, B., Spaduti, O., Yama- abhai, M. et al. (2010) Characterisation of recombinant pyranose oxidase from
the cultivated mycorrhizal basidiomycete *Lyophyllum shimeji* (hon-shimeji). Microb. Cell Fact. 9, 57.

[22] Hallberg, B.M., Leitner, C., Haltrich, D. and Divine, C. (2004) Crystal structure of the 270 kDa homotetrameric lignin-degrading enzyme pyranose 2-oxidase. J. Mol. Biol. 341, 781–796.

[23] Bannwarth, M., Heckmann-Pohl, D., Bastian, S., Giffhorn, F. and Schulz, G.E. (2006) Reaction geometry and thermostable variant of pyranose 2-oxidase from the white-rot fungus *Peniophora* sp. Biochemistry 45, 6589–6595.

[24] Johnsrud, S.C. and Eriksson, K.E. (1985) Cross-breeding of selected and mutated homokaryotic strains of *Phanerochaete chrysosporium* K-3 – new cellulase deficient strains with increased ability to degrade lignin. Appl. Microbiol. Biotechnol. 21, 320–327.

[25] Savitsky, P., Bray, J., Cooper, C.D.O., Marsden, B.D., Mahajan, P., Burgess-Brown, N.A. et al. (2010) High-throughput production of human proteins for crystallization: the SGC experience. J. Struct. Biol. 172, 1–13.

[26] Doyle, S.A. (2005) High-throughput cloning for proteomics research. Methods Mol. Biol. 310, 107–113.

[27] Kabsch, W. (1993) Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. J. Appl. Crystallogr. 26, 795–800.

[28] McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C. and Read, R.J. (2007) Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674.

[29] Adams, P.D., Afonine, P.V., Bunkócz, G.V., Chen, B., Davis, I.W., Echols, N. et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D 66, 213–221.

[30] Murshudov, G.N., Vagin, A.A. and Dodson, E.J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D 53, 240–255.

[31] Collaborative Computational Project. (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D 50, 760–763. (Number 4).

[32] Painter, J. and Merritt, E.A. (2006) Optimal description of a protein structure in terms of multiple groups underlying TLS motion. Acta Crystallogr. D 62, 439–450.

[33] Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard, M. (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A 47, 110–119.

[34] Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D 60, 2126–2132.

[35] DeLano, W.L. (2002) The PyMOL Molecular Graphics System. Palo Alto, CA, USA: DeLano Scientific.

[36] Kujawa, M., Ehner, H., Leitner, C., Hallberg, B.M., Prongjit, M., Sucharitakul, J. et al. (2006) Structural basis for substrate binding and regioselective oxidation of monosaccharides at C-3 by pyranose 2-oxidase. J. Biol. Chem. 281, 35104–35115.

[37] Vetrani, C., Maeder, D.L., Toliday, N., Yip, K.S.P., Stillman, T.J., Britton, K.L. et al. (1998) Protein thermostability above 100 °C: A key role for ionic interactions. Proc. Natl. Acad. Sci. USA 95, 12300–12305.

[38] Lebbink, J.H.G., Knapp, S., van der Oost, J., Rice, D., Ladenstein, R. and de Vos, W.M. (1999) Engineering activity and stability of *Thermotoga maritima* glu- tamate dehydrogenase. II: Construction of a 16-residue ion-pair network at the subunit interface. J. Mol. Biol. 289, 357–369.

[39] Gerk, L.P., Leven, O. and Müller-Hill, B. (2000) Strengthening the dimerisation interface of Lac Repressor increases its thermostability by 40 °C. J. Mol. Biol. 299, 805–812.

[40] Karshikoff, A. and Ladenstein, R. (2001) Ion pairs and the thermotolerance of proteins from hyperthermophiles: a ‘traffic rule’ for hot roads. Trends Biochem. Sci. 26, 550–556.

[41] Bell, G.S., Russell, R.J.M., Connaris, H., Hough, D.W., Danson, M.J. and Taylor, G.L. (2002) Stepwise adaptations of citrate synthase to survival at life’s extremes. Eur. J. Biochem. 269, 6250–6260.

[42] Thomas, A.S. and Elcock, A.H. (2004) Molecular simulations suggest protein salt bridges are uniquely suited to life at high temperatures. J. Am. Chem. Soc. 126, 2208–2214.

[43] Constantini, S., Colonna, G. and Facchiano, A.M. (2008) ESBRJ: a web server for evaluating salt bridges in proteins. Bioinformatics 3, 137–138.

[44] Shinde, U. and Inouye, M. (1993) Intramolecular chaperons and protein folding. Trends Biochem. Sci. 18, 442–446.

[45] Gold, M.H. and Alic, M. (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Mol. Biol. Rev. 57, 605–622.

[46] Ritch, V.J. Jr, Nipper, T.G., Akleswaran, L., Smith, A.J., Pribnow, D.G. and Gold, M.H. (1991) Lignin peroxidase from the basidiomycete *Phanerochaete chrysosporium* is synthesized as a preproenzyme. Gene 107, 119–126.

[47] De Boer, H.A., Zhang, Y.Z., Collins, C. and Reddy, C.A. (1987) Analysis of nucleotide sequences of two ligninase CDNAs from a white-rot fungus, *Phanerochaete chrysosporium*. Gene 60, 93–102.

[48] Nishimura, I., Okada, K. and Koyama, Y. (1996) Cloning and expression of pyra- nose oxidase cDNA from *Coriolus versicolor* in *Escherichia coli*. J. Biotechnol. 52, 11–20.

[49] Takakura, Y. and Kuwata, S. (2003) Purification, characterization, and molecular cloning of a pyranose oxidase from the fruit body of the basidiomycete, *Tricholoma matsutake*. Biosci. Biotechnol. Biochem. 67, 2598–2607.

[50] Nordahl Petersen, T., Brunak, S., von Heijne, G. and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

[51] Kaplur, P.A. and Diederichs, K. (2012) Linking crystallographic model and data quality. Science 336, 1030–1033.

[52] Lovell, S.C., Davis, I.W., Arendall, W.B., I, de Bakker, W.B., Word, J.M., Prisant, M.G. et al. (2003) Structure validation by Cα geometry: 4, ψ and Cβ deviation. Prot. Struct. Func. Genet. 50, 437–450.