First Crystal Structure of a Fungal High-redox Potential Dye-decolorizing Peroxidase

SUBSTRATE INTERACTION SITES AND LONG-RANGE ELECTRON TRANSFER*

Received for publication, July 12, 2012, and in revised form, December 11, 2012 Published, JBC Papers in Press, December 12, 2012, DOI 10.1074/jbc.M112.400176

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Background: DyP-type peroxidases catalyze biotechnologically important reactions.

Results: Based on the crystal structure of a fungal DyP, the conformational flexibility of Asp-168 is elucidated. Tyr-337 is identified as a surface-exposed substrate interaction site.

Conclusion: Asp-168 and Tyr-337 are key residues directly involved in AauDyPI-catalysis.

Significance: Peroxidases are biocatalysts, much sought after and ubiquitous enzymes in nature.

Dye-decolorizing peroxidases (DyPs) belong to the large group of heme peroxidases. They utilize hydrogen peroxide to catalyze oxidations of various organic compounds. AauDyPI from Auricularia auricula-judae (fungi) was crystallized, and its crystal structure was determined at 2.1 Å resolution. The mostly helical structure also shows a β-sheet motif typical for DyPs and Cld (chlorite dismutase)-related structures and includes the complete polypeptide chain. At the distal side of the heme molecule, a flexible aspartate residue (Asp-168) plays a key role in catalysis. It guides incoming hydrogen peroxide toward the heme iron and mediates proton rearrangement in the process of Compound I formation. Afterward, its side chain changes its conformation, now pointing toward the protein backbone. We propose an extended functionality of Asp-168, which acts like a gatekeeper by altering the width of the heme cavity access channel. Chemical modifications of potentially redox-active amino acids show that a tyrosine is involved in substrate interaction. Using spin-trapping experiments, a transient radical on the surface-exposed Tyr-337 was identified as the oxidation site for bulky substrates. A possible long-range electron transfer pathway from the surface of the enzyme to the redox cofactor (heme) is discussed.

Peroxidases (EC 1.11.1) are ubiquitous enzymes that catalyze the oxidative conversion of various compounds utilizing hydrogen peroxide (H₂O₂) as electron acceptor. As a common trait, most peroxidases, although phylogenetically unrelated, contain a heme B (iron protoporphyrin IX) molecule as the redox cofactor.

A division of this heterogenic group led to the formation of four families, one of which consist of the dye-decolorizing peroxidases or DyPs (EC 1.11.1.19) (1). According to Welleinder’s systematics, which were later on extended (2, 3), DyPs were initially grouped in the class of secretory fungal peroxidases viz. class II of the peroxidase-catalase superfamily. This class further comprises lignin peroxidase, manganese peroxidase, and versatile peroxidase. However, DyPs turned out to be phylogenetically as well as structurally unrelated to all hitherto described peroxidase families. As a result, a new family of enzymes was established to accommodate these unusual peroxidases (4). From a structural viewpoint, DyPs are best considered members of a highly diverse superfamily of proteins comprising, among others, Clds and DyPs, sharing a ferredoxin-like core of β-sheets as a common feature (5). Actually, even the term “DyP” circumscribes a polyphyletic group that can be roughly divided into four different entities. The groups DyPA–C comprise predominantly bacterial enzymes, whereas DyPD is a fungal group (6). The first DyP was discovered in 1995 (7). A mixture of extracellular enzymes secreted by the basidiomycete Bjerkandera adusta (the strain was back then misidentified as Thanatephorus cucumeris) was reported to efficiently oxidize anthraquinone dyes such as Reactive Blue 5 (7, 8). The enzyme responsible for the decolorization process was characterized as a heme peroxidase of unusual chemical properties. Despite the general versatility of peroxidase chemistry, no fungal peroxidases had been known to efficiently oxidize synthetic anthraquinone dyes so far.

DyPs catalyze many reactions, among them several conversions that are biotechnologically desirable. Enzymatic assays on a fungal DyP from the basidiomycete Auricularia auricula-judae (AauDyP, formerly labeled “AjP”) have been reported in a
Catalytic Features of a DyP-type Peroxidase

The putative oxidative cycle of fungal DyPs is largely equivalent to that in other peroxidases, but was recently extended by the proposal of a swinging mechanism of a distal aspartate residue during Compound I formation (12). In Scheme 1, the formation of Compound I is depicted. In a first step, H₂O₂ enters the heme cavity of the enzyme in resting state where it displaces a water molecule that occupies the sixth ferric iron coordination site of the protoporphyrin IX system. A distal basic amino acid residue mediates the rearrangement of a proton in H₂O₂. In peroxidases, this base typically is a histidine, whereas in DyPs, this key residue is substituted by an aspartate residue (see “Results and Discussion”). The heme molecule is then oxidized to the radical-cationic state where it displaces a water molecule that occupies the proximal heme ligand, usually a histidine residue.

The above reactions summarize the general catalytic cycle of peroxidases. The term “Enzyme” indicates the peroxidase resting state, and XH₂ and XH⁺ are a substrate molecule and the corresponding radical species, respectively.

Up to the present, only a few DyP-like enzymes from fungi have been described biochemically in detail, and only one crystal structure has been published so far (14). Research on bacterial members has been comparatively more extensive. A native crystal structure was needed to substantiate recent results involving fungal as well as bacterial DyP-type peroxidases (5, 12). To gain further insight into the structural properties of the eucaryotic (in this case fungal) DyPs, AauDyPI from the jelly fungus A. auricula-judae (Auriculariales, Basidiomycota) was purified and crystallized, and the crystal structure was determined at 2.1 Å resolution (Protein Data Bank (PDB) code 4AU9).

EXPERIMENTAL PROCEDURES

Isolation of Nucleic Acids, PCR, and mRNA Sequencing—For detailed methodology, see elsewhere (49). In short, mRNA was extracted from mycelia of A. auricula-judae grown in agitated cultures. For cDNA synthesis, the total mRNA (1 µg) was primed and subsequently reverse-transcribed. Afterward the obtained cDNA was amplified following a customized PCR protocol.

Primers were designed to amplify fragments of a DyP-type peroxidase gene from A. auricula-judae. The obtained PCR products were purified and sequenced. Strain DSMZ 11236 (from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was checked for species identity by internal transcribed spacer PCR (ITS-PCR) on genomic DNA with primers ITS1 and ITS4 (15).

Protein Production and Purification of AauDyPI—Production and purification of AauDyPI were carried out as described in a previous publication (9). To summarize, A. auricula-judae was grown in agitated tomato juice medium at 24 °C. At maximum peroxidase activity level, the cultures were harvested and directly filtrated. The enzyme was purified using Q-Sepharose chromatography columns. Purified enzyme was stored in 5 mM sodium acetate buffer at 4 °C.

Crystallization—Purified AauDyPI was crystallized using protein concentrations of 10.7 mg ml⁻¹ in 5 mM sodium acetate, pH 6.8. Various crystallization kits (Hampton Research, Aliso Viejo, CA and Jena Bioscience, Jena, Germany) were used for initial screens. Crystals were grown at 19 °C by the hanging drop vapor diffusion method with a 1:1 (v/v) ratio of protein-to-precipitant in 4-µl drops. Intergrown plate-like crystals were obtained with a precipitant solution consisting of 0.17 M sodium acetate trihydrate, 0.085 M Tris hydrochloride, pH 8.5, 25.5% (w/v) polyethylene glycol 4.000, and 15% (v/v) glycerol. From these crystal clusters, very small single fragments of about

SCHEME 1. General formation of Compound I in peroxidases. This is the key step in heme peroxidase catalysis. After proton rearrangement by the distal base-catalytic residue, two electrons are transferred from heme to hydrogen peroxide, leading to the active state of the enzyme, called Compound I (13). B indicates a basic residue, e.g., a histidine or aspartate/glutamate in peroxidases. L indicates the proximal heme ligand, usually a histidine residue.
Catalytic Features of a DyP-type Peroxidase

0.2 × 0.1 × >0.001 mm were retrieved that seemed suitable for x-ray crystallographic experiments. To verify the results of structure determination, we examined the purified protein via LC-MS.

Data Collection and Processing—AauDyPI crystals were directly flash-cooled in liquid nitrogen. X-ray diffraction data were collected on the macromolecular crystallographic beamline ID14-4 at the European Synchrotron Radiation Facility (ESRF) (Grenoble, France) (16). Data were indexed, processed, and scaled with XDS (17, 18). Data collection and processing statistics are given in Table 1.

Structure Determination—The structure of AauDyPI was determined by molecular replacement using MOLREP (19) and Phaser (20). An ad interim structure of the B. adusta DyP (PDB code 2D3Q) was used as search model. This structure will be referred to as BadDyP in the following. Side chains of nonconserved amino acids were truncated at the last carbon atom common to the target and model residues with the CCP4-program CHAINSAW (21). Because there are two molecules per asymmetric unit, density modification including noncrystallographic symmetry averaging was performed to improve the initial phases using the CCP4 program PARROT (22). An initial model was built with Coot (23) and refined in REFMAC5 (24). Further improvement was achieved by successive cycles of model building and refinement.

Identification of a Redox-active Surface-exposed Tyrosine—Chemical modification of AauDyPI was carried out as described by Inokuchi et al. (25) and Miki et al. (26). In brief, AauDyPI was incubated with 5–150-fold excess of N-bromosuccinimide or tetratinromethane, respectively. The modified proteins were directly used in an enzyme assay monitoring the oxidation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) at 420 nm. Spin trapping was performed using the protocol of Zhao et al. (27). At first AauDyPI was deglycosylated using 10 units of peptide-N-glycosidase F (Aldrich) at room temperature for 1.5 days. The thus prepared enzyme was mixed with excess proline nitric oxide after incubation with peroxycetic acid. This mixture was incubated for 15 min at 37 °C and then rebuffered in 20 mM K3PO4 buffer, pH 7.2. The enzyme was recovered from the reaction mixture by SDS-PAGE and subsequent Coomassie Blue staining. The bands were cut from the gel using a sterile scalpel. Prior to mass spectrometry, the protein was digested in-gel with elastase and thermolysin, respectively. The peptide containing the spin-trapped amino acid residue was identified using an Agilent 6520 quadrupole-TOF mass spectrometer coupled to a reverse-phase HPLC unit and a nano-electrospray ionization source.

Electron Transfer Calculations, Probing of the Heme Access Channel, and Imaging—The calculation of electron transfer pathways was performed with the program HARLEM (as described in Ref. 28) using the PATHWAY module. Volume and shape of the heme access channel were calculated with HOLLOW (29).

Two coordinate files of AauDyPI were created from the original file, each one containing one of the two conformers of Asp-168. Solvent molecules were removed from the coordinate files, and a simulation of the Compound I state containing a randomly placed guaiacol (2-methoxyphenol) molecule was built using Coot. An oxygen atom was thus placed distally perpendicular to the heme plane at 1.7 Å distance from iron as found in the Compound I structure of horseradish peroxidase (PDB entry 1HCH (30)). Ligand docking was then performed with the Molegro Virtual Docker (MVD) (31). The ligand binding cavities were identified using the Expanded van der Waals algorithm with a grid size of 0.5 Å. 50 docking runs were performed using the guaiacol molecule as ligand. The population size was set to 200 over a radius of 12 Å around the predicted binding cavity with a grid size of 0.2 Å. 10,000 iterations per position were performed. Clustering of similar positions was enabled using an r.m.s. deviation of 1.5 Å. The ligand positioning was constrained to the predicted binding site, i.e. the distal heme cavity. Images were created in PyMOL (32) and CCP4mg (33).

RESULTS AND DISCUSSION

Structure Solution and Refinement—Improvement of crystal size and quality of AauDyPI proved unexpectedly arduous. Despite numerous variations of the initial crystallization condition, only very thin plate-like crystals were obtained. Crystal growth was exclusively observed in solutions with a high concentration of polyethylene glycol (PEG) 4000. The widely used ammonium sulfate yielded only amorphous precipitate. The presence of cryoprotectants such as glycerol or 2-methyl-2,4-pentanediol ab initio was beneficial to crystal quality. Additives such as various amino acids did not show any positive effects. Bearing in mind the size and morphology of the crystals, surprisingly good x-ray diffraction data were collected; the best crystals diffracted to a maximum resolution of about 2.1 Å (Table 1).

The relatively high sequence identity between AauDyPI and BadDyP (PDB code 2D3Q) suggested molecular replacement as a promising approach to solve the structure. Indeed, this technique was successful. Phase modification techniques gave a further improvement, resulting in a high-quality electron density map. This allowed us to build an initial model of the complete protein, which was subsequently refined in REFMAC5. The final refinement statistics are supplied in Table 1.

Overall Structure of AauDyPI—AauDyPI is a globular glycoprotein with a helical basic architecture and a prominent β-sheet motif spanning the distal side of the heme plane. A proximal N- and a distal C-terminal domain embed the heme molecule, which itself is partly flanked by the β-sheet of the ferredoxin-like fold. Like other DyPs, it is structurally very distinct from the classical heme peroxidases because the latter are practically completely helical. The monomeric AauDyPI has an ellipsoid-like shape with dimensions of 70 × 42 × 40 Å3. The refined model of AauDyPI consists of the complete polypeptide chain of 448 amino acids as predicted by gene sequencing. Even the C and N terminus could be modeled as well as some rather large flexible loop regions (Fig. 1A), where electron density was mostly poorly defined. In the crystal, there are two molecules per asymmetric unit. The Cα-r.m.s. between the two subunits is 0.375 Å, which is in the order of the estimated coordinate error of 0.257 Å. Only in the flexible surface loop regions were larger deviations between the two molecules observed. Given the consensus sequence Asn-X-Ser/Thr with X being any amino acid except proline or aspartic acid, there are four poten-
Catalytic Features of a DyP-type Peroxidase

Tional glycosylation sites. Three of them could be verified crystallographically, whereas closer inspection of Asn-322 reveals that this fourth site is inaccessible for glycosyltransferases and consequently is not glycosylated. The glycosylation sites are exclusively distributed on the “lower” end of the proximal side with respect to the heme molecule (Fig. 1A), especially at large, protruding loops that are susceptible to proteolytic attacks. Therefore, the carbohydrate moieties might have a protective function against proteases.

In AauDyPI, some carbohydrate chains are definitely glycosylated to a higher extent than outlined here. Residual electron density, albeit weak and difficult to interpret, strongly indicates to a higher extent than outlined here. Residual electron density against proteases.

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TABLE 1
Summary of data collection and refinement statistics of AauDyPI

| Data collection       | Values in parentheses belong to the highest resolution shell. |
|-----------------------|---------------------------------------------------------------|
| Space group           | P2₁                                                          |
| Unit-cell parameters (Å) | a = 66.60; b = 46.69; c = 141.20; β = 91.35 | |
| Beamline              | ID14-4, ESRF                                                  |
| Wavelength (Å)        | 0.9395                                                       |
| Temperature (K)       | 100                                                          |
| No. of crystals       | 1                                                            |
| Resolution range (Å)  | 49.01–2.10 (2.23–2.10)                                       |
| Total no. of reflections | 131,951 (19,754)                                             |
| No. of unique reflections | 49,651 (7,661)                                              |
| Completeness (%)      | 96.9 (93.4)                                                  |
| Rmerge (I)            | 11.02 (3.35)                                                 |
| Rfree                  | 0.094 (0.437)                                                |

| Reﬁnement            | Values in parentheses belong to the highest resolution shell. |
|-----------------------|---------------------------------------------------------------|
| Rmerge,Rfreea         | 0.180/0.261                                                   |
| Molecules per asymmetric unit | 2 | |
| Solvent content (%)   | 42                                                            |
| No. of amino acid residues | 896 | |
| No. of heme molecules | 2                                                            |
| No. of carbohydrates  | 8                                                            |
| No. of ligands        | 6 glycerol, 1 acetate, 1 Tris                                |
| No. of water molecules| 679                                                          |
| r.m.s. deviations     | Bond lengths (Å) 0.011                                        |
| Bond angles (°)       | 1.25                                                         |
| Ramachandran plot (%) | Most favored 93.9                                        |
| Most favored          | Disallowed 5.4                                                |
| Allowed               | Disallowed 0.7                                               |
| Average B factors (Å²) | Main chain atoms 24.3                                       |
| Side chain atoms      | Water molecules 37.1                                          |
| Ligands/hemes/carbohydrates | Overall 36.1                                           |

a Rfree values are calculated with 5% of the data.

FIGURE 1. Overview of the AauDyPI structure. A, ribbon diagram of AauDyPI. The ferredoxin-like β-sheet fold is highlighted in green, the protoporphyrin IX system is in dark violet with the central iron atom as a tan sphere, and the carbohydrate chains with their corresponding N-linked asparagine residues are in purple. The chain termini are labeled as well. B, superposition of BadDyP (mauve and purple) onto AauDyPI (green shading). The Ca trace is shown with helices as tubes. C, superposition of Clb (pink) onto AauDyPI (whitish and green shading). The Ca trace is shown with helices as tubes.
ring D (Fig. 2A). In DyPs, the propionate at pyrrole C is tilted into an unusual conformation due to the formation of strong hydrogen bonds to the protein (Fig. 2B). Propionate D of AauDyPI forms a long H-bond with Nη2 of Arg-311 and additionally with four water molecules. This hydrogen-bonding pattern allows propionate D to maintain an unstrained conformation. In contrast, propionate C is much more confined by five hydrogen bonds. Among them, there are three strong H-bonds to the main chain N of Ile-170 and Ala-171 and to Nη2 of Arg-332, respectively. Additionally, two more H-bonds to Ne of Arg-255 and to one water molecule are formed. This strong hydrogen-bonding network obviously forces propionate C into a high-energy conformation, hence enforcing a rather close distance of 3.6 Å between one of the carboxyl oxygens and a methine group of the porphyrin ring. As a result, the sp²-plane of the propionate lies almost orthogonally to the heme plane. The oxygen sensor FixL might be another example where interactions with arginine side chains constrain the propionate chains into unusual geometries upon O₂ binding (38).

Geometry of the Distal Heme Cavity and Implications for Catalysis—A first partial catalytic cycle for DyPs has been proposed by Sugano et al. (4). Fundamentally, the catalytic cycle in DyPs is the same as in other peroxidases. The heme molecule is oxidized by peroxides to the radical-cationic oxoferryl species Compound I, which is two electrons deficient from the resting state. One electron is abstracted from the iron, resulting in Fe(IV), and another one is abstracted from the porphyrin ring. It became apparent that a distal acid-base pair is essential for Compound I formation in peroxidases to rearrange a proton in the peroxide substrate. When compared with classical peroxidases, proton rearrangement or abstraction in DyPs is accomplished in a different way. Because binding to the heme iron lowers the pKₐ of hydrogen peroxide (39), deprotonation does not require any strong bases. Most peroxidases use a histidine residue, whereas in DyPs, an even weaker base, a deprotonated aspartate residue, is employed. An arginine is paired with the histidine or aspartate, respectively. It does not take part in the rearrangement process directly, but is essential for coordinating H₂O₂ at the sixth ligand site of heme-Fe(III) and stabilizing Compound I. Surprisingly, the aspartate residue is not essential for catalysis in procaryotic DyPs. Although Compound I stability is reduced in mutants lacking said aspartate, they retain a good portion of their enzymatic activity. Mutation of the distal arginine, however, results in complete failure to form Compound I and, concomitantly, loss of any peroxidase activity (40).

The mechanism of Compound I formation in DyPs has been amended recently by the concept of a swinging mechanism of the distal aspartate residue during this process (12). Said aspartate changes the location of its side chain upon the presence of cyanide, a structural analog of hydrogen peroxide, in the distal heme cavity when compared with the cyanide-free enzyme. It was proposed that this aspartate swings toward the heme molecule in the presence of H₂O₂ and thereupon mediates the rearrangement of a proton. Once Compound I formation is finished, it swings back to the initial position.

In AauDyPI, Asp-168 plays the part of the mediating residue. During model building, some issues arose concerning this key amino acid. Electron density clearly indicated two distinct conformations of Asp-168 as shown in Fig. 3.

The Oδ1 and Oδ2 atoms of Asp-168 form hydrogen bonds to Nη1 of Arg-332, thus establishing the catalytic distal acid-base pair. This is the conformation necessary for Compound I formation. In the alternative conformation (refined with 0.5 occupancy), the side chain of Asp-168 has flipped toward a loop region in the opposite direction. The flipping angle is about 130°. Now both Oδ1 and Oδ2 are able to form hydrogen bonds to the protein backbone, more precisely to the carbonyl oxygen of Gly-160. Additionally, each carboxyl oxygen is bridged to a water molecule by a hydrogen bond of 3 Å. Average temperature factors of <30 Å² for the atoms of Asp-168 in each conformation in both subunits create further confidence in the probability of our model. These data are consistent with two distinct conformations of the Asp-168 side chain, indicating flexibility in a peptide, which is otherwise rather rigid and crystallo-
Catalytic Features of a DyP-type Peroxidase

Identification of Substrate Oxidation Sites—To explore the possibility of small substrates being oxidized directly in the heme cavity, we tentatively modeled a Compound I state of AauDyPI. As described above, Compound I was modeled based on the parameters of the oxoferryl species reported for horseradish peroxidase (PDB entry 1HCH 27). A guaiacol molecule was included in this hypothetical structure, a standard substrate for peroxidases. The MolDock algorithm of MOLEGR0 was used to examine whether the distal substrate binding pocket was large enough to accommodate the model compound. The calculations confirmed that molecules of this size narrowly fit in the heme access channel of the enzyme.

However, the majority of DyP substrates are considerably larger than guaiacol. Keeping in mind the nature of Compound I (involving formation of a radical cation) and widening the heme access channel features an overall funnel-like shape that significantly narrows toward the distal site of the heme (see Fig. 5). It is largely hydrophobic with the exception of the distal Asp-168/Arg-332 pair and several hydrophilic residues directly at the channel entry. In BadDyP, the opening to the binding pocket was reported to measure about 3 Å, having a roughly circular appearance (Fig. 4A). The above mentioned Asp-168 is part of a strictly conserved tetrad that defines the distal substrate binding pocket. In one conformation, the side chain of Asp-168 coincides with that of Asp-171 in BadDyP. Consequently, AauDyPI features a very similar opening with a slightly ellipsoid appearance (Fig. 4B). These are the general conformations involved in forming Compound I. Fig. 4C shows the opening to the substrate binding pocket with Asp-168 in its alternative conformation facing toward the peripheral protein backbone. In this situation, the opening is enlarged significantly, measuring about 6 Å in one dimension and about 3 Å in the other. Switching the side chain toward the carbonyl oxygen of Gly-160, possibly after Compound I formation, Asp-168 might work like a gatekeeper that allows passage of substrate molecules bigger than hydrogen peroxide (i.e. small organic compounds). Consequently, this would enable the enzyme to oxidize substrates directly inside the heme cavity.

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idue (in AauDyPI, there are seven tyrosines in total), spin-trapping experiments were performed followed by mass spectrometric analysis. Excess proline nitric oxide was used to yield nitrotyrosine from transient tyrosyl radicals (27); it generates NO, which acts as an in situ radical scavenger trapping the expected tyrosyl radicals that were generated by adding peroxycetic acid to an AauDyPI solution. Nitrosotyrosine thus formed is subsequently oxidized to the stable nitrotyrosine. The modified enzyme was finally digested proteolytically and subjected to LC-MS analysis. Nitrotyrosine was solely detected at residue 337.

Tyr-337 is surface-exposed and located at the beginning of a large protruding loop comprising 20 amino acids. Its side chain is partially embedded in a hollow formed by side chains of residues Leu-149 and Gln-218 and the main chain of residues 354–358 with the edge of the aromatic ring pointing toward the solvent. The latter peptide is the end of the aforementioned loop and extends into the inner β-sheet, which is closest to the distal site of the heme. Tyr-337 forms hydrogen bonds via backbone amide nitrogen of Leu-357 involving a through-space jump of 3.3 Å followed by the leucine side chain and another jump (3.8 Å) from Cβ1 of Leu-357 to one of the pyrrole rings. The effective length of this pathway is about 13 Å. The hydroxyl group Tyr-337 forms a strong hydrogen bond of 2.6 Å with the carboxylate of Glu-354. It has been reported that acidic side chains in the vicinity of a tryptophan or a tyrosine stabilize an emerging radical cation (26, 47, 48). Alignments with other DyPs show that Tyr-337 is a conserved residue in fungal as well as bacterial DyPs and the related enzymes TyrA from Shewanella oneidensis and EfeB from Escherichia coli but is missing in Cld-like proteins (5).

Our experiments provide the first unambiguous identification of a surface-exposed oxidation site in the large group of dye-decolorizing peroxidases. Further investigations including crystallization of AauDyPI-substrate complexes and site-directed mutagenesis to further substantiate the significance of Tyr-337 and the suggested double responsibility of Asp-168 for the catalytic cycle are in progress.

Acknowledgments—We gratefully acknowledge the opportunity to collect diffraction data on the synchrotron beamline ID14-4 at the ESRF. We thank the staff for technical support. Dr. Eric Haaf of the proteomics unit of the Zentrum für Biosystemanalyse (ZBSA) in Freiburg, Germany is thanked for LC-MS experiments.

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Catalytic Features of a DyP-type Peroxidase

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