(Format No. 3)

SUMMARY OF DOCTORAL THESIS

Name: **ODUOR HENRY JOSEPH OGOLA**

Title: **Insight on Dye-decolorizing Peroxidase Family (DyP-type Peroxidases) from the Molecular Analysis of a Novel Bacterial DyP from *Anabaena* sp. Strain PCC7120**

(*Anabaena* sp. strain PCC7120 株由来新規バクテリア DyP の分子的解析から色素脱色型ペルオキシダーゼファミリーDyP-タイプペルオキシダーゼの見解)

Dye decolorizing peroxidases (DyP), a novel family of heme-containing peroxidases named after their capability to efficiently oxidize the high redox potential trichromatic anthraquinoic dyes (AQ), have been identified in various basidiomycetes and bacterial species. DyPs have been the focus of significant interest due to their high specificity for AQ, low pH optima, dramatic differences in their active site topology, heme-binding motif and structural divergence from classical plant superfamily peroxidase counterparts. Furthermore, the involvement of DyPs in the alternative biocatalytic degradation system of recalcitrant methoxylated aromatics in the lignin polymeric moiety, catalysis of enantioselective sulfoxidation, enzymatic production of "bioflavors" from carotenoids, and in detergents and food-bleaching applications have also been documented, thereby illustrating the huge potential applications of DyPs. The current understanding on DyPs is largely based on a plant pathogenic fungus *Thanetophorous cucumeris* Dec1 DyP homolog which has been characterized extensively, and very little information is available on other members of the DyP family. In particular, studies on bacterial DyPs have only been limited within the automatically translated sequence or structural data. To further understand the structure-function relation and potential application of the novel DyP-type enzymes in general, this thesis focused on the less known bacterial DyP members. This study cloned and characterized a novel putative bacterial DyP homolog (here named AnaPX) in a photosynthetic

cyanobacterium *Anabaena* sp. PCC7120 genome.

Bioinformatic analysis revealed that the open reading frame alr1585 of *Anabaena* sp. PCC 7120 encodes a heme-dependent peroxidase (AnaPX) belonging to the novel DyP-type peroxidase family (EC 1.11.1.X), putatively grouped together in Class D DyPs due to their higher homologies to fungal DyP homologs. The *alr1585* gene was cloned and the active form of the enzyme heterologously expressed in *Escherichia coli.* The purified enzyme was a 53-kDa tetrameric protein with a pI of 3.68, a low pH optima (pH 4.0), and an optimum reaction temperature of 35°C. Biochemical characterization revealed an iron protoporphyrin-containing heme peroxidase with a broad specificity for aromatic substrates such as guaiacol, 4-aminoantipyrine and pyrogallol. The enzyme efficiently catalyzed the decolorization of AQ dyes like Reactive Blue 5, Reactive Blue 4, Reactive Blue 114, Reactive Blue 119 and Acid Blue 45 with decolorization rates of 262, 167, 491, 401, and 256 $\mu M \cdot min^{-1}$, respectively. The apparent K_m and k_{cat}/K_m for Reactive Blue 5 were 3.6 μM and 1.2×10^7 $M^{-1}s^{-1}$, respectively, while the apparent K_m and k_{cat}/K_m values for H_2O_2 were 5.8 μM and 6.6×10^6 $M^{-1}s^{-1}$, respectively. In contrast, the decolorization activity of AnaPX toward azo dyes was relatively low, but was significantly enhanced 2~50-fold in the presence of the natural redox mediator; syringaldehyde. Notably, AnaPX showed relatively higher activities towards both guaiacol and RB5 compared to *T. cucumeris* Dec1 DyP that shows only higher specificity towards RB5 than guaiacol. Homology modeling and docking simulation indicated that guaiacol easily access the active site within the heme pocket of AnaPX, while in fungal DyP, a constricted heme pocket entrance provides steric hinderance limiting accessibility, and the guaiacol molecule binds within hydrogen bond distant outside the heme catalytic site. The broad specificity and catalytic efficiency for hydrogen donors and synthetic dyes showed the potential application of AnaPX as a useful alternative of horseradish peroxidase (HRP) or fungal DyPs.

Biochemical characterization and stability analysis also revealed that the AnaPX is sensitive to sub-millimolar H_2O_2 concentrations, and exhibits a biphasic inactivation profile consisting of an initial compound III formation leading to heme degradation and eventual protein denaturation. Site-directed mutagenesis to substitute 5 Met residues with high redox residues Ile, Leu, or Phe was performed for the improvement of the

enzyme stability towards H_2O_2. Results indicated that the heme cavity mutants M401L, M401I, M401F and M451I had significantly increased H_2O_2 stability of 2.4-, 3.7-, 8.2- and 5.2-fold, respectively. Surprisingly, the M401F and M451I retained 16% and 5% activity at 100 mM H_2O_2, respectively, in addition to maintaining high dye-decolorization activity towards AQ and azo dyes at 5 mM H_2O_2 and showing a slower rate of heme degradation than the WT AnaPX. The correlation between the k_{cat} values with the hydrogen peroxide stability clearly showed that the enzymatic activity decreases was concomitantly associated with the stability increases. This behavior supports the mechanism-based inactivation process as a higher turnover due to high production of the free radicals results to a higher oxidative self-inactivation. The observed stabilization of AnaPX were attributable to the replacement of potentially oxidizable Met residues either increasing the local stability of the heme pocket or limiting of the self-inactivation electron transfer pathways due to the above mutations. The increased stability of AnaPX variants coupled with the broad substrate specificity can be potentially useful for the further practical application of these enzymes especially in bioremediation of industrial wastewater contaminated with recalcitrant AQ.

In contrast to typical peroxidases that possess a generally conserved distal histidine critical for compound I intermediate formation in peroxidase catalysis, DyPs have a distal aspartate residue forming the absolutely conserved novel GXXDG motif instead. To clarify the functional and structural role played by distal Asp as a general acid-base catalyst playing similar mechanistic role of distal His in traditional peroxidase reaction mechanism, several distal Asp^{204} variants (D204A, D204N and D204H) were developed. Interestingly, steady-state kinetics analysis of all the mutants showed significantly depressed catalytic efficiency (k_{cat} /K_m) towards the peroxidase substrates studied, only D204H showing significant catalysis. UV absorption and rapid scan stopped-flow spectroscopy indicated that D204A and D204N inactivity was due to mutants inability to form compound I reaction intermediate in presence of H_2O_2. The formation rate of compound I (k_{1app}) for WT AnaPX (1.18×10^7 $M^{-1}s^{-1}$) were approximately 10^3 times faster than that for D204H mutant (k_{1app} = 6.2 x 10^4 $M^{-1}s^{-1}$) both exhibiting a pH dependence characteristic of titration distal Asp The pK_a of Asp204 could be estimated directly (pK_a ~ 4.3) from the WT AnaPX data indicating its deprotonation for efficient reaction with H_2O_2; while acid-base transition pK_a observed for D204H (pK_a ~3.9) could be assigned as arising from titration of the His residue.

EPR analyses also suggested that both WT AnaPX and D204H were able to form compound I radical that rapidly delocalized to the protein matrix as protein-based radical species, these intramolecular electron transfer being efficient in WT AnaPX. Also inactive low spin heme iron species observed in EPR analyses contributed to the D204H depressed activity relative to WT AnaPX. The observed low catalytic efficiency of D204H mutant could be attricbuted ; 1) the presence of inactive low spin species; 2) the lack of hydrogen bond to fix distal His in optimal position for the stable ternary complex formation; and, 3) the disturbance of substrate access and binding in the heme active site pocket due to mutation of 204 position, therefore depressing k_{1app} and eventually enzyme catalysis. Therefore, factors such steric relationship between the distal ligand, hydrogen-bonding network within the distal cavity and conformational dynamics of the heme pocket in addition to nature of distal residue, are important in controlling the enzyme peroxide reactivity. Collectively, these results provide evidence that Asp^{204} residue is an essential catalytic residue for peroxidase activity in AnaPX, suggesting that redox step in compound I formation is the rate-limiting step in the catalysis of reducing substrates in DyPs.