Engineering of the H_2O_2-binding pocket region of a recombinant manganese peroxidase to be resistant to H_2O_2

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Abstract The manganese peroxidase produced by *Phanerochaete chrysosporium*, which catalyzes the oxidation of Mn^{2+} to Mn^{3+}, is easily inactivated by the hydrogen peroxide (H_2O_2) presented in the reaction. We attempted to increase H_2O_2 resistance by the conformational stabilization around the H_2O_2-binding pocket. Based on its structural model, engineering of oxidizable Met273 located near the pocket to a non-oxidizable Leu showed a great improvement. Furthermore, after treatment at 1 mM H_2O_2 where the wild-type is completely inactivated, full activity can be retained by engineering the Asn81, which might have conformational changes due to the environment of the pocket, to a non-bulky and non-oxidizable Ser. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Manganese peroxidase; Chemical oxidation; Hydrogen peroxide

1. Introduction

Manganese peroxidase (MnP) catalyzes the oxidation of Mn^{2+} to Mn^{3+} utilizing hydrogen peroxide (H_2O_2) and forms complexes with an organic acid. The Mn^{3+}–chelate complex is a highly reactive non-specific oxidant [1] capable of oxidizing a variety of environmental pollutants [2,3]. This powerful oxidant has been proposed to be important for the degradation of sterically bulky compounds that are unable to gain access to the active site of a peroxidase. This mediated system of degradation is potentially valuable for some applications, such as in the pulp and paper industries, the degradation of synthetic polymers and the degradation of environmental pollutants. However, MnP is very sensitive to inactivation by H_2O_2 or thermal treatment. An attempt to increase the thermo-stability of MnP has been reported [4], but one to increase the resistance to H_2O_2 has not been reported. The inactivation pathway by H_2O_2 of MnP has been studied and the formation of compound III results in heme bleaching and irreversible inactivation [5]. One of the primary causes of protein instability is susceptibility to conformational change around the active site and subsequent inactivation or denaturation. These phenomena have been especially reported for proteins containing methionine, cysteine and tryptophan residues, by oxidation in or around the active site [6–8]. In this study, we examined the crystal structure of MnP and performed site-directed mutagenesis of the native MnP gene to create a recombinant MnP with conversion of unstable amino acid residues around the H_2O_2-binding region to stable amino acid residues. We have already constructed a two-stage reaction system for pulp bleaching involving native MnP [9]. Our ultimate goal is to produce a more resistant enzyme, which would be suitable for use in the presence of a high concentration of H_2O_2 for commercial applications.

2. Materials and methods

2.1. Chemicals

H_2O_2 was purchased from Wako Chemicals (Japan). GSSG (glutathione oxidized form), ABESF (4-(2-aminoethyl)-benzenesulfonyl fluoride), oxalic acid, IPTG (isopropyl-thio-β-D-galactoside), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), ampicillin were purchased from Sigma.

2.2. Structural modeling of MnP

We constructed models of MnP isozyme 2 and mutants by the homology modeling method using the LOOK and SEGMOD module of GeneMine [10]. Crystal structure files for MnP isozyme 1 were obtained from the Brookhaven Protein Data Bank (1MNP), and used as the backbone of our starting structure. The addition of the necessary hydrogen atoms to the structure was performed by means of the biopolymer module of Insight II 97.0. Simulations were performed with the Discover 3.0.0. program (Molecular Simulations Inc., USA) with energy minimization and molecular dynamic calculations. Non-bonded parameters were obtained by the Cell Multipole method [11] under the Esff force field. Molecular dynamic calculations were performed with the NVT ensemble at 296 K.

2.3. Construction of an Escherichia coli expression vector and production

On the basis of the gene sequence of MnP isozyme 2 (GenBank, PHAMNP2A; [12]), we amplified MnP isozyme 2 cDNA from *Phanerochaete chrysosporium* ATCC64314 mRNA by the RACE PCR method. The amino acid sequence of the cloned MnP was slightly different from PHAMNP2A as follows: 98N→D and 126L→M. MnP gene was amplified by PCR to introduce a *Nde*I site at the start codon and a *Hin*dIII site immediately downstream of the gene. The *Nde*I–*Hin*dIII cut fragment was inserted into *E. coli* expression vector, pET21b(+) (Novagen) [13]. In the resulting plasmid pET21b(+)-MnP, the MnP gene is under the control of the T7 promoter, possesses a C-terminal six-His tag, and the ampicillin marker. pET21b(+)-MnP and mutants were transformed into *E. coli* strain BL21(DE3)LysS (Novagen), and then grown at 37°C in Luria broth to an absorbance of 0.6 at 600 nm. IPTG was added to a final concentration of 0.1 mM, followed by harvesting by centrifugation. The cell pellets were resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0, 10 mM dithiothreitol (DTT), 1 mM EDTA), and then frozen overnight. After thawing, 1 mM AEBSF and 0.5% Triton X-100 were added, followed by sonication for 20 min. After centrifugation, the pellets containing inclu-

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Abbreviations: MnP, manganese peroxidase; H_2O_2, hydrogen peroxide

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sion bodies were resuspended in solubilization buffer (8 M urea, 50 mM Tris–HCl, pH 8.0, 2 mM EDTA, 1 mM DTT) overnight.

2.4. Purification and refolding of the recombinant MnP

The 8 M urea solution was applied to a His-tag column (Pharmacia), which was washed with washing buffer (8 M urea, 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 10 mM imidazole), and then the protein was eluted with elution buffer (200 mM imidazole in washing buffer). The purity was checked by SDS–PAGE and estimated to be around 95%.

The eluted sample was diluted in 2 M urea containing 50 mM Tris–HCl, pH 8.0, 5 μM hemin, 0.7 mM GSSG and 1 mM $CaCl_2$. The refolding mixture was stored at 4°C in the dark overnight. Refolded preparations of recombinant MnP were dialyzed exhaustively against 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$.

2.5. H_2O_2 stability and dependency studies

MnP solution was incubated in the presence of 0–3.0 mM H_2O_2 at 37°C for 60 min, and then diluted to 0.1 mM H_2O_2. MnP activity (Mn^{2+} to Mn^{3+}) was measured using the general peroxidase substrate ABTS [14]. The concentrations used were 0.5 mM ABTS, 2 mM oxalate, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2 and 25 mM sodium succinate, pH 4.5, and measured at 405 nm for 5 min.

Enzyme mixtures containing various concentrations of H_2O_2 (0.006–10 mM) were used for H_2O_2 dependency studies. MnP activity was measured as described above. All data are the mean values for at least four samples.

3. Results and discussion

3.1. Engineering the methionine residues of MnP

Chemical oxidation of a methionine residue to a sulfoxide derivative has been reported for some proteins [6–8]. H_2O_2 treatment leads to the inactivation of enzymes that is correlated directly with the production of methionine sulfoxide. MnP has seven methionines in its molecule. Fig. 1 shows the positions of the engineered Met of the structural model of the cloned MnP. Among them, Met67, 237 and 273 were located within 12 Å from the center of the active site, and

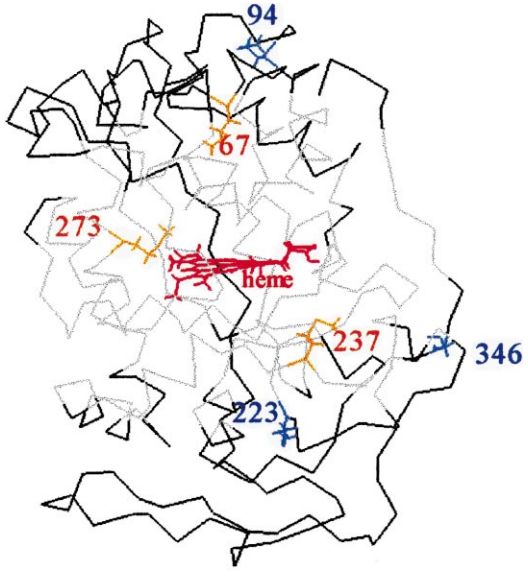

Fig. 1. Structural mapping of the substituted methionine residues. Substitution of three methionine residues (67, 237 and 273; orange) located within a radius of 12 Å (gray) from the heme, the resulting enzymes being designated as IMnP. Another three methionine residues (94, 223 and 346; blue), whose side chains were located on the surface of the molecule, were also substituted with leucine, the resulting enzymes being designated as OMnP.

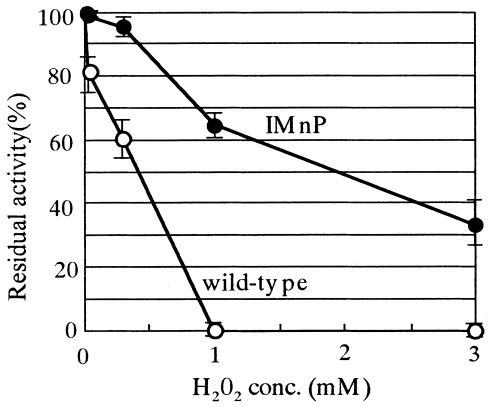

Fig. 2. Oxidative stability of IMnP. Enzyme mixtures were incubated in the presence of various concentrations of H_2O_2 at 37°C for 60 min, and then the residual activity of MnP was measured. ○, wild-type; ●, IMnP (substitution of inner methionine residues; M67L, M237L and M273L). All data are the mean values for at least four samples.

Met94, 223 and 346 were located on the surface region of the MnP molecule. Predictions based on data on homologous exchange of amino acids [15] suggest that Leu or Val is the most suitable for the substitution of a Met. Four of them are the components of the B′, C, J helix structure, and Leu is one of the most suitable for the form action of a helix structure, so we chose Leu for the substitution of Met.

We engineered two mutants, one was engineered at the inner three methionines (IMnP; M67L/M237L/M273L), which would prevent the conformational changes near the active site caused by chemical oxidation, and the other was engineered at the outer three residues, whose side chains were directed toward the outer region (OMnP; M94L/M223L/M346L). Also, the side chain of one more, Met265, was buried in the molecule, so we did not substitute this position.

IMnP and wild-type were incubated at 37°C in the presence of 0–3 mM of H_2O_2 for 60 min, the residual activities being shown in Fig. 2. IMnP retained more than 60% of its initial activity at a concentration of 1 mM H_2O_2 and more than 30% at a concentration of 3 mM H_2O_2, while wild-type was completely inactivated at a concentration of 1 mM H_2O_2.

In order to produce a more resistant enzyme for commercial applications, we evaluate the H_2O_2 resistance of MnP by measuring MnP activity in the presence of various concentrations of H_2O_2. The H_2O_2 dependency of IMnP and OMnP is shown in Table 1. IMnP showed excellent H_2O_2 resistance, being 50% stable with 1.13 mM H_2O_2 for 5 min. Wild-type was 50% stable with 0.18 mM H_2O_2, so that H_2O_2 dependency of IMnP was 6.3-fold higher than that of the wild-type. OMnP showed almost the same dependency with the wild-type. We concluded that the engineering of inner Met was the critical method for improving both of the stability and the dependency as to H_2O_2, because of protection of the oxidative environment around the active site. While the engineering of the surface Met (OMnP) was not effective.

To determine which Met is most critical for the H_2O_2 resistance, we engineered three single mutants (M67L, M237L and M273L). These mutants were reacted in the presence of various concentrations of H_2O_2 (Table 1). Among the mutants, only the M273L showed high H_2O_2 resistance, i.e. 4.1-fold higher than that of wild-type. Met273 is the nearest residue to the active site. It is located about 4.3 Å from the

Table 1
Improvement of the H_2O_2 resistance of various mutants

Clone No.	Amino acid No.[a]							H_2O_2 dependency		H_2O_2 stability[d]
	67	81	94	223	237	273	346	H_2O_2 (mM)[b]	Mutant/wild-type[c]	Residual activity (%)
Wild-type	M	N	M	M	M	M	M	0.18 ± 0.1	1.0	0
N81S	–	S	–	–	–	–	–	1.0 ± 0.0	5.5	99.6 ± 0.7
OMnP	–	–	L	L	–	–	L	0.22 ± 0.1	1.2	6.8 ± 4.2
IMnP	L	–	–	–	L	L	–	1.13 ± 0.1	6.3	64.2 ± 4.4
M273L	–	–	–	–	–	L	–	0.74 ± 0.2	4.1	–
M237L	–	–	–	–	L	–	–	0.18 ± 0.1	1.0	–
M67L	L	–	–	–	–	–	–	0.25 ± 0.1	1.4	–
IN81S	L	S	–	–	L	L	–	0.72 ± 0.1	4.0	46.8 ± 2.7

[a]The amino acid substitutions in each mutant are shown.
[b]The H_2O_2 concentrations which exhibited 50% relative activity are shown.
[c]Mutant/wild-type ratio of H_2O_2 concentrations which exhibited 50% relative activity.
[d]% residual activity after treatment for 60 min at 37°C in the presence of 1 mM H_2O_2.

heme edge and faces the active site pocket, so this residue is solvent-accessible and oxidized easily. M67 and M237 are located 6.6 Å and 12 Å from the heme edge. These residues are near the active site, but are buried in the MnP molecule, so they are not solvent-accessible. Our data suggested that the conversion of oxidizable methionine residue around the pocket to non-oxidizable residue was critical for improvement of the oxidative resistance.

3.2. Engineering the conformational stability of the H_2O_2-binding pocket of MnP

Fig. 3 shows the structural model of the H_2O_2-binding pocket of cloned MnP, in which H_2O_2 was directly supported by His46 and Arg42. At least four amino acid residues, Ser78, Ala79, Asn80 and Asn81, are located near the H_2O_2-binding site. Among them, Ser78 and Ala79 are non-oxidizable and non-bulky amino acids, and Asn80 is one of the conserved amino acids which comprise the hydrogen bond network (His46–Asn80) of MnP [16]. This H-bond between the distal His and Asn80 is also conserved in plant and fungal peroxidases, and ensures that the distal His is available for accepting a proton from the peroxide. Asn81 (indicated in red) is not a conserved amino acid residue in other peroxidases. In addition, protein inactivation by conformational changes depending on deamidation at asparaginyl residues has been reported [17,18], so that Asn81 might have the conformational changes in the environment around the pocket. At this position, a hydrophilic amino acid is conserved in other MnPs. So we engineered Asn81 to hydrophilic, non-bulky and non-oxidizable Ser, whose –OH functional group might be form a hydrogen bond with the main peptide chain.

N81S retained more than 99% of its initial activity at a concentration of 1 mM H_2O_2 for 1 h, while wild-type was completely inactivated (Table 1). H_2O_2 dependency of N81S was 5.5-fold higher than that of the wild-type. Also we engineered a mutant designated as IN81S (M67L/N81S/M237L/M273L), which contained both the IMnP and N81S mutations. The synergistic effect of mutations on IN81S variant was not observed (Table 1).

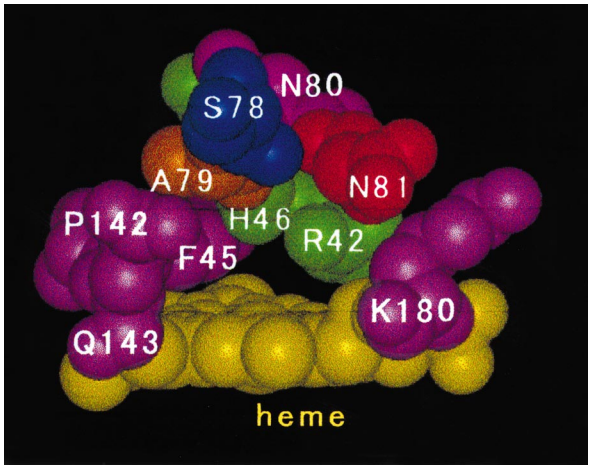

Fig. 3. Structural model of H_2O_2-binding pocket of wild-type. The residues of H_2O_2-binding site are His46 and Arg42 (green) in the distal heme pocket. The residues around the pocket are 78 (blue), 79 (orange), 80 (pink), 81 (red), and others (light pink), and heme (yellow), with CPK (Corey–Pouling–Keltun) space filling.

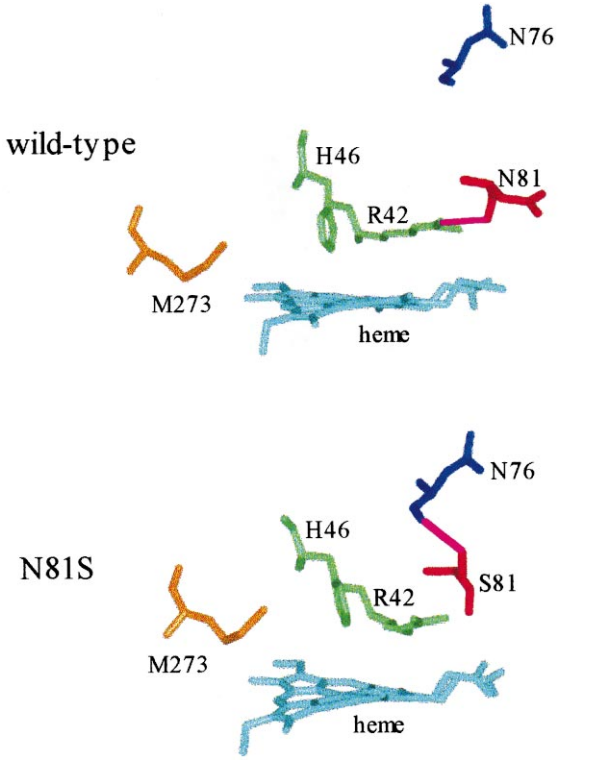

Fig. 4. Structural models of the H_2O_2-binding pocket of mutants. The side chains of the residues are 42, 46 (green), 81 (red), 273 (yellow) and 76 (blue), and the heme (sky blue). The hydrogen bond interactions between 81, and 42 and 76 are shown as pink lines.

Structural models of the wild-type and N81S were obtained by computer modeling (Fig. 4). In N81S, substitution of serine caused a hydrogen bond interaction between the –OH group of Ser81 and the main peptide chain of Asn76. This suggested that the hydrogen bond interactions might cause conformational stabilization. This structure–function consideration might explain why N81S exhibited higher H_2O_2 resistance.

4. Conclusion

We suggested that prediction of critical restricted mutations in the functional region, using a structural model generated with a computer-driven model-building system or an X-ray crystal structure study, is an effective method for converting the function of the protein efficiently. In this study, we constructed models of MnP isozyme 2 and predicted oxidizable and/or solvent-accessible and/or conformationally unstable amino acid residues around the H_2O_2-binding pocket. All mutants characterized are shown in Table 1. We investigated the effects of engineering of oxidizable Met facing the pocket to non-oxidizable residues (IMnP, M273L and IN81S), also the conformational stabilization around the pocket (N81S and IN81S) was proven to be very effective for improvement of the H_2O_2 resistance.

Native MnP was purified on a DEAE-Sepharose gel from *P. chrysosporium* by the same method as that of [19]. The native MnP carried sugar chains on the surface of the molecule [20], while recombinant MnP did not. In this study, in spite of lacking sugar chains, recombinant MnP showed almost the same H_2O_2 resistance as native MnP (data not shown). In addition, the engineering of an oxidizable Met located on the surface region is not so effective. This suggested that amino acid residues around the pocket critically affected the H_2O_2 resistance.

We examined the effectiveness of site-directed mutagenesis of critical residues around the functional region. These strategies would be applicable for the engineering of other H_2O_2-binding pocket-containing proteins for improvement of the H_2O_2 resistance. Moreover, fine-tuning for accommodation by the pocket of MnP would require a random mutation process such as the directed evolution method. On the other hand, the present results will be useful for industrial applications, such as in the pulp and paper industries and the degradation of environment pollutants.

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