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Modification of the heme active site to increase the peroxidase activity of thermophilic cytochrome P450: A rational approach

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ABSTRACT

The site specific mutants of the thermophilic P450 (P450 175A1 or CYP175A1) were designed to introduce residues that could act as acid–base catalysts near the active site to enhance the peroxidases activity. The Leu80 in the distal heme pocket of CYP175A1 was located at a position almost equivalent to the Glu183 that is involved in stabilization of the ferryl heme intermediate in chloroperoxidase (CPO). The Leu80 residue of CYP175A1 was mutated with histidine (L80H) and glutamine (L80Q) that could potentially form hydrogen bond with hydrogen peroxide and facilitate formation and stabilization of the putative redox intermediate of the peroxidase cycle. The mutants L80H and L80Q of CYP175A1 showed higher peroxidase activity compared to that of the wild type (WT) CYP175A1 enzyme at 25 °C. The activity constants (k_{cat}) for the L80H and L80Q mutants of CYP175A1 were higher than those of myoglobin and wild type cytochrome b562 at 25 °C. The optimum temperature for the peroxidase activity of the WT and mutants of CYP175A1 was ~70 °C. The rate of catalysis at temperatures above ~70 °C was higher for L80Q mutant of CYP175A1 compared to that of the well known natural peroxidase, horseradish peroxidase (HRP) that denatures at such high temperature. The peroxidase activities of the mutants of CYP175A1 were maximum at pH 9, unlike that of HRP which is at pH ~5. The results have been discussed in the light of understanding the structure–function relationship of the peroxidase properties of these thermostable heme proteins.

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1. Introduction

Heme proteins play diverse roles in biological systems [1,2]. These proteins having the same heme prosthetic group are responsible for many different types of functions such as oxygen storage and transport, electron transfer, oxidation and oxygenation of different substrates etc. The functional diversity of the heme in different heme proteins has been attributed partly to the differences in the amino acid environment around the distal heme pocket [1]. It however, still remains a great challenge to understand the molecular basis of the specific function of the heme in a particular type of heme protein environment [3,4]. The most common strategy for the understanding of the structure–function relationship for an enzyme involves the substitution of amino acid residues near the active site by site-directed mutagenesis followed by investigations of the effect of the substitution on the function of that enzyme [3,5–11].

Peroxidases are heme-enzymes catalyzing the one electron oxidations that use hydrogen peroxide as the electron acceptor and have interesting biocatalytic properties with potential use in biosensing and immunoassays [12,13]. The peroxidase catalytic cycle generally involves the formation of the high valent reactive intermediates (Scheme 1 known as the compound I (oxo-ferryl porphyrin(P) π-cation radical, (P+•)Fe(IV) O, CpdI) and the compound II (oxo-ferryl porphyrin, (P)Fe (IV) O, CpdII) that are responsible for their catalytic activity [12,14,15]. The reducing substrate (AH) is generally converted into a radical ($A^{\bullet}$), which subsequently forms dimeric or polymeric products. These reactive intermediates are suggested to be generated by the heterolytic cleavage of the O–O bond of hydroperoxy ferric heme iron. The catalytic cycle and the peroxide shunt pathway of the heme monooxygenases (P450 enzymes) have also been proposed to involve the highly reactive intermediate (compound I) [14]. The X-ray crystal structure analyses of these heme containing enzymes such as chloroperoxidase (CPO, PDB: 1CPO.pdb), horseradish peroxidase (HRP, PDB: 1ATJ.pdb), cytochrome *c* peroxidase (CcP, PDB: 2CYP.pdb) and other peroxygenases (some P450s like $P450_{bs\beta}$) suggest the presence of certain key residues at the distal heme pocket, which enhance the generation of compound I species by acting as acid–base catalysts and by stabilizing it as well by forming hydrogen bond with the ferryl oxygen [4,16]. A histidine is one of such important amino acid residue around the distal heme pocket that is found to be highly conserved in many heme-peroxidases including HRP [3]. The His42 and Arg38 present near the distal side of the heme in HRP are reported to be responsible for the very high rate of compound I formation as well as for the high activity of the enzyme [17–20]. In contrast to HRP, the distal Glu183 in CPO is shown to be the crucial residue to act as the acid base catalyst [19,21,22]. The cytochrome P450,

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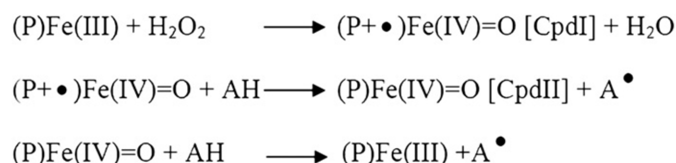

Scheme 1. Formation of high valent reactive intermediates in peroxidase catalytic cycle. P: corresponds to protoporphyrin IX inside the protein matrix. P+•: indicates porphytin cation radical. AH is reducing substrate and A• is substrate radical. CpdI and CpdII refer to compound I and compound II redox intermediates.

whose natural function is monooxygenation of substrates, lacks such architecture of the distal pocket [14]. The kinetics of generation of compound I by hydrogen peroxide in cytochrome P450 was proposed to be very slow possibly because of the absence of appropriate amino acids at the distal pocket of the heme that could act as acid–base catalyst in stabilizing this reactive intermediate [23,24].

Extensive studies on the peroxidase activities of different heme proteins have been reported in literature and modification of certain distal residues have been shown to enhance the peroxidases activity of hemeproteins such as myoglobin, cytochrome *c* etc. [8,9,20,25,26]. One of the interests in the study of the peroxidase activity of different heme enzymes also stems from the extensive use of peroxidases as biocatalysts for food processing and bioremediation [13,27]. The low thermal stability is one of the disadvantages of many such commercially available enzymes in the industry. Therefore, the peroxidases with high thermal stabilities are highly preferred over the mesophilic enzymes. Several peroxidases isolated from thermophilic organism showed very high thermostability but they were shown to lose the activity in the presence of H_2O_2 [28–30]. In the recent years, efforts have been made to improve the peroxidase activity in both mesophilic and thermophilic proteins by rational protein engineering but most of them often fail to retain their activity at higher temperatures [3,5,8,26]. Efforts have also been made to enhance the thermostability of HRP [31,32] by modification of the enzyme.

The CYP175A1 is a 44-kDa soluble thermostable cytochrome P450 from *Thermus thermophilus* HB27 [33]. This enzyme was shown to hydroxylate β-carotene at 3- and 3′-positions, resulting in β-cryptoxanthin and zeaxanthin, although its natural substrates are still not known [34,35]. The crystal structure of CYP175A1 (PDB: 1N97.pdb) shows absence of suitable amino-acid residues in the distal heme pocket that could act as acid–base catalysts as in the heme peroxidases such as HRP and CPO. The aim of the present project is to modify the active site of this thermostable P450 to introduce a residue at the distal heme pocket that could act as an acid–base catalyst and thus enhance the peroxidase activity in the mutant enzyme. This could potentially lead to the creation of a thermally stable artificial peroxidase. In this project we have carried out molecular modeling studies and identified that the Leu80 residue in the CYP175A1 could be suitably mutated to enhance the peroxidase activity of the mutant enzyme. The high thermostability (T_m = 87 °C) of CYP175A1 has the advantage to engineer this protein to create an artificial thermostable enzyme [33]. We have studied the peroxidase activities of L80H and L80Q mutants of the enzyme at different temperatures and pH to determine the optimum conditions and results were compared with those of the archetype peroxidase, HRP. The substitution of the leucine with histidine or glutamine was found to enhance the peroxidase activity of CYP175A1, which is more prominent at high temperature.

2. Experimental procedures

2.1. Materials

Phenyl sepharose and Sephadex G-25 columns were from GE Healthcare Bio-Sciences AB, Uppsala. Hydroxyapatite material was from Biorad Laboratories Inc, Hercules, CA., ammonium sulphate, sodium cholate, guanidine hydrochloride (GdnHCl), ampicillin sodium salt, isopropyl β-D-thiogalacto-pyranoside (IPTG), lysozyme, phenyl methyl sulphonyl fluoride (PMSF), oligonucleotide primers, guaiacol, ABTS were purchased from Sigma Chemicals Co. Restriction enzymes was purchased from New England Biolabs (NEB). DNAse I was purchased from Boehringer Mannheim, Germany. Amplex Red was purchase from Invitrogen. Hydrogen peroxide (H_2O_2) was purchased from Qualigen, India. The H_2O_2 stock solution was freshly prepared every time and its concentration was quantified spectrophotometrically (ε_{240} = 39.4 M^{-1} cm^{-1}) [36]. All buffer components, solvents and other reagents were of analytical grade. Deionized water (Millipore) was used in the preparation of all the buffers.

2.2. Mutation of recombinant CYP175A1

The pKK-223 plasmid, encoding the wild type CYP175A1 enzyme was a kind gift from Prof. Vlada B. Urlacher (University of Stuttgart, Germany). Site-directed mutagenesis was carried out using Quikchange™ mutagenesis kit (Stratagene Co.). The forward and reverse oligonucleotide primers were GAGGGGtCTC-XXX-ACCGACTGGGGG and CCCCCCAGTCGGT-YYY-GAGaCCCCTC respectively. Where XXX and YYY were the codons for the 80th residue in forward and reverse primer. The mutants were designed by changing the sequences of XXX and YYY in the primers, e.g., with XXX = CaC for L80H, Cag for L80Q, gaC for L80D, gag for L80E, atC for L80I, and with YYY = GtG for L80H, ctG for L80Q, Gtc for L80D, ctc for L80E, Gat for L80I mutation. These primers introduced a *BsaI* restriction site at 239 bp position of the structural gene along with the desired mutation.

2.3. Expression and purification of wild type and variant CYP175A1

The wild type, L80I, L80H and L80Q mutant CYP175A1 proteins were expressed and purified by a small modification of the reported method [34]. pKK-223 plasmid having structural gene for the expression of CYP175A1 was transformed in the BL21 (DE3) codon plus RP cells and grown overnight in 5 ml 2xYT medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. The overnight culture was used to inoculate 700 ml 2xYT medium keeping the same concentration of antibiotics and was followed by further incubation at 37 °C and 170 rpm. Induction was done by adding 1 mM IPTG to the cell culture after reaching OD_{600nm} ~ 0.8–1 and incubated for 50 h at 30 °C at 130 rpm. The cell pellets were harvested by centrifugation 6000 rpm for 15 min.

The cell pellets were suspended in 10 volumes of lysis buffer (50 mM Tris–HCl: pH 7.5, 200 mM KCl, 1 mM EDTA, 0.2 mM PMSF, 1 mg/ml lysozyme, DNase I: 40 Units/ml and 2–3 mM $MgCl_2$) and stirred for 45 min at 4 °C. The cell suspension were sonicated and then centrifuged at 19,000 rpm for 90 min. The supernatant was treated with slow addition of solid $(NH_4)_2SO_4$ to reach 35% saturation with stirring at 4 °C for 45 min followed by centrifugation at 18,000 rpm for 45 min. The collected supernatant was further proceeded to reach 50% $(NH_4)_2SO_4$ saturation followed by centrifugation at the same condition (at 18,000 rpm for 45 min). After ammonium sulfate fractionation, the pellet was dissolved in minimum volume of 50 mM Tris–HCl buffer (pH 7.5) which was centrifuged and filtered (0.4 μm). The filtrate was loaded to a phenyl sepharose column, pre-equilibrated with 35% saturated $(NH_4)_2SO_4$ in 50 mM Tris–HCl buffer (pH 7.5). The column was washed with the same buffer as above and then subjected to a linear gradient (35–0%) of $(NH_4)_2SO_4$ in 50 mM Tris–HCl buffer (pH 7.5). Elution was continued with 50 mM Tris–HCl buffer (pH 7.5) having 1% sodium cholate. The protein fractions having R_z (A_{418}/A_{280}) ≥ 1 were combined, concentrated with centricon tube (Millipore) and loaded onto a hydroxyapatite column. CYP175A1 enzyme was eluted from the column by applying linear gradient of 50–300 mM potassium phosphate (pH 7.4) containing 5% glycerol. The L80H mutant protein was again purified by gel filtration chromatography (Superdex 75 prep grade, Pharmacia Biotech).

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