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Research paper

Site-directed mutagenesis of tobacco anionic peroxidase: Effect of additional aromatic amino acids on stability and activity

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ABSTRACT

Tobacco anionic peroxidase (TOP) is known to effectively catalyze luminol oxidation without enhancers, in contrast to horseradish peroxidase (HRP). To pursue structure-activity relationship studies for TOP, two amino acids have been chosen for mutation, namely Thr151, close to the heme plane, and Phe140 at the entrance to the active site pocket. Three mutant forms TOP F140Y, T151W and F140Y/T151W have been expressed in *Escherichia coli*, and reactivated to yield active enzymes. Single-point mutations introducing additional aromatic amino acid residues at the surface of TOP exhibit a significant effect on the enzyme catalytic activity and stability as judged by the results of steady-state and transient kinetics studies. TOP T151W is up to 4-fold more active towards a number of aromatic substrates including luminol, whereas TOP F140Y is 2-fold more stable against thermal inactivation and 8-fold more stable in the reaction course. These steady-state observations have been rationalized with the help of transient kinetic studies on the enzyme reaction with hydrogen peroxide in a single turnover regime. The stopped-flow data reveal (a) an increased stability of F140Y Compound I towards hydrogen peroxide, and thus, a higher operational stability as compared to the wild-type enzyme, and (b) a lesser leakage of oxidative equivalents from TOP T151W Compound I resulting in the increased catalytic activity. The results obtained show that TOP unique properties can be further improved for practical applications by site-directed mutagenesis.

1. Introduction

Peroxidases catalyze oxidation of numerous native and artificial electron donors with hydrogen peroxide (Scheme 1), where S and P are the one-electron substrate and its oxidation product.

Peroxidases, especially horseradish peroxidase (HRP), are widely used in analytical biotechnology due to easy photometric (3,3′,5,5′-Tetramethylbenzidine) or luminescent (luminol in enhanced chemiluminescence) detection of the oxidation products in the picomolar range.

In this laboratory we isolated and characterized another promising peroxidase for applied purposes, anionic tobacco peroxidase (TOP), and developed a robust protocol for the production of recombinant wild-type enzyme. The study of native tobacco peroxidase [1] revealed a number of unusual properties. High stability of TOP in the wide range of pH allows its activity towards veratryl alcohol to be detected at pH < 2 [2], where horseradish peroxidase becomes inactive at once. Both native and wild-type recombinant TOP are extremely active towards luminol in the absence of chemiluminescence enhancers, and thus, the enzyme is a perfect label for chemiluminescent assays [3,4]. The entrance to the heme-binding pocket in TOP is screened by Glu141-Gln149 pair providing the additional stability against dissociative inactivation of the enzyme [5], in contrast to HRP, which has no such stabilization pair. The previously reported introduction of

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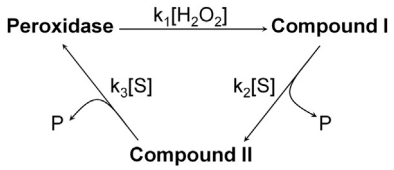

Scheme 1. The peroxidase catalytic cycle begins with rapid interaction between the enzyme and hydrogen peroxide, yielding the so-called Compound I that contains two oxidative equivalents, the first one on the heme iron (oxyferrylheme) and the second one, a free radical (a *p*-cation radical on the porphyrin ring). Compound I is reduced to the ferric enzyme via intermediate formation of Compound II.

Glu143 into HRP resulted in the increased stability at the expense of enzyme activity [6]. Based on the published observations, we conclude that enzyme stability is controlled by the easiness of heme dissociation, i.e. solvent accessibility to the heme, which is directly linked to the enzyme activity.

Most plant peroxidases contain only one conserved tryptophan residue [7,8], which is indirectly involved in catalytic cycle [9]. Formation of tryptophan and tyrosine radicals has been observed in myoglobin [10–14], cytochrome *c* peroxidase [15–17], and HRP [18]. The single Trp117 in HRP may participate in electron transfer through protein [19] and Tyr185 is important for oxidation of aromatic compounds. The existence of two distinct substrate interaction sites was proposed for lignin peroxidase, i.e. a heme-edge site typical for all peroxidases and a novel site centered at Trp171 required for the oxidation of veratryl alcohol [20]. Two TOP mutant forms, Q116W and L157W, with additional Trp residues at the enzyme surface at the site opposite to the active center entrance (Fig. 1) were constructed and characterized previously in this laboratory: introduction of Trp led to changes in kinetic parameters in reaction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), (ABTS) oxidation [21].

In this work, TOP structure-activity relationship studies were continued with the emphasis on the role of aromatic residues introduced at the enzyme surface close to the active site. We have chosen Thr151 located at the enzyme surface opposite to the entrance, but exactly in the heme plane, and Phe140 by the entrance to the active center for Trp and Tyr substitutions, respectively (Fig. 1). The double mutant form TOP F140Y/T151W has been constructed as well. The reason for our choice is the well-known ability of Trp and Tyr residues to participate in electron transfer reactions and the existence of the intramolecular electron transport chain in peroxidases perfectly illustrated by the

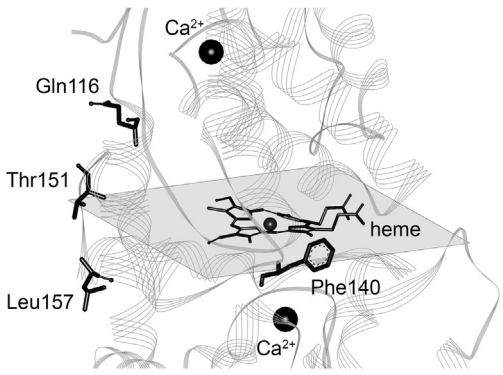

Fig. 1. Location of substituted amino acid residues in tobacco peroxidase molecule. * Residues Gln116 and Leu157 were substituted for tryptophan in previous work [21].

established fact of direct electron transfer between peroxidase and graphite or gold electrodes [22–26].

We expected the introduced mutations to affect the catalytic properties of the enzyme, possibly creating the variants better performing in the reactions of practical significance.

2. Materials and methods

2.1. Materials

Potassium ferrocyanide, luminol, guaiacol, phenol, 4-aminoantipyrine, 2,2′-azino-bis(3-ethylbenzthiasoline-6-sulfonate) (ABTS), ortho-phenylene diamine (o-PD), p-iodophenol, oxidized glutathione, dithiotreitol, urea, Tris, calcium chloride, hemin, sodium acetate, sodium phosphate, potassium hydroxide, hydrogen peroxide were from Sigma–Aldrich (St. Louis, MO). All reagents purchased were of the highest purity available.

2.2. Site-directed mutagenesis

The gene of anionic tobacco peroxidase was cloned in the laboratory of Prof. L.M. Lagrimini [2]. Site-directed mutagenesis was performed with Quik-Change kit (Stratagene) using following primers:

T151W:

T151W_fwd: 5′ – CAC AAT TCT GGA ATA AGG GGA TG – 3′,
T151W_rev: 5′ – CCC CTT ATT CCA GAA TTG TGG TAT – 3′.

F140Y

F140Y_fwd: 5′ – CCT AGC CCC TAT GAA ACA CTT GCT GTA – 3′,
F140Y_rev: 5′ – TAC AGC AAG TGT TTC ATA GGG GCT AGG – 3′.

TOP F140Y/T151W was constructed on the basis of T151W plasmid using the primers introducing F140Y mutation. The mutation efficiency was 100% as confirmed by sequencing the plasmids from three individual clones.

2.3. Growth conditions and protein expression

Wild-type TOP was expressed in *Escherichia coli* BL21(DE3) CodonPlus cells as we described before [26]. TOP mutants were produced by the same protocol as for wild-type TOP, except the cultivation temperature was lowered. This resulted in a 10% increase in the enzyme yield. Transformed *E. coli* BL21(DE3)CodonPlus cells were grown in 600 mL LB-medium prepared with 10 mM Tris–HCl buffer, pH 8.0, containing 0.04 mg/mL kanamicin at 31 °C. Expression was induced with 2×10^{-4} M IPTG in the mid-log phase of cell growth, and the cultivation was continued for 6 h at 27 °C. The protein was synthesized in the form of inclusion bodies, and the expression level was ca. 40% of the total protein. The biomass was collected by centrifugation at 5000 g.

2.4. Protein refolding and purification

The biomass was resuspended in 10 mM Tris–HCl buffer, pH 8.0, and disrupted by sonication (22 kHz, 10 min) in the presence of 2 M NaCl and 10 mM DTT. The mixture was incubated for 1.5 h at RT. The supernatant was removed, and the precipitate was washed with 50 mM Tris–HCl buffer, pH 8.5, with subsequent solubilization in 60 mL of 6 M urea containing 1 mM DTT. The solubilized TOP apoprotein (95% purity, 1 mg/mL) was added drop by drop to 600 mL of the refolding medium and incubated at 4 °C. The conditions for refolding of mutants were the same as for the wildtype