Biochemical and Biophysical Research Communications **262,** 297–301 (1999)
Article ID bbrc.1999.1155, available online at http://www.idealibrary.com on IDEAL®

Tryptophanless Recombinant Horseradish Peroxidase: Stability and Catalytic Properties

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Received June 11, 1999

The tryptophanless mutant of horseradish peroxidase, W117F, has been constructed and expressed in *Escherichia coli.* The mutation affects enzyme folding and stability. The optimum composition of the refolding medium requires the presence of ammonium sulfate. The yield of mutant is ca. 8000 U per liter of the optimized refolding medium with the specific activity of 1100–1500 U/mg (compared to 25,000 U per liter and 2000 U/mg for the recombinant wild-type enzyme). The mutant is more stable in acid media, in the reaction course and toward irradiation. The effect of hydrogen peroxide pretreatment on radiation-induced inactivation of the wild-type and mutant enzyme indirectly indicates participation of Trp-117 in electron transfer pathways through the enzyme molecule. This is in agreement with the steady-state kinetic data interpreted in terms of Trp-117 participation in electron transfer within the Michaelis complex.

Key Words: **horseradish peroxidase; site-specific mutagenesis; Trp117Phe; refolding; stability; enhanced chemiluminescence; radiation-induced inactivation; steady-state kinetics.**

Horseradish peroxidase (HRP) belongs to the superfamily of heme-containing plant peroxidases (EC 1.11.1.7) and it is the most studied enzyme among classic plant peroxidases due to its numerous analytical applications. The enzyme catalyses a three-step process of oxidation of a donor substrate with hydrogen peroxide (1):

$$E + H_2O_2 \rightarrow EI + H_2O(k_1) \quad [1]$$

$$EI + S \rightarrow EII + S^{\cdot}(k_2) \quad [2]$$

$$EII + S + 2H^+ \rightarrow E + S^{\cdot} + H_2O(k_3), \quad [3]$$

where E is ferric enzyme, EI and EII are the oxidized intermediates, Compounds I and II, respectively, and S and $S^{\cdot}$ are the electron donor substrate and the radical product of its one-electron oxidation. The reduction of Compound II with a donor substrate is usually the rate-limiting step in peroxidase catalysis (1). In the absence of donor substrates HRP Compound I quickly converts into Compound II and subsequently to the native ferric enzyme. It is likely that the role of a donor substrate in this case is played by the protein itself, i.e., the active heme center is reduced with concomitant oxidation of protein amino acid residues. The higher is the Compound II/ferric enzyme redox potential, the less stable it is. Thus, for Asn70Val and Asn70Asp HRP mutants with the increased redox potential, Compound II can be detected only at high pH (2). The rapid conversion of cytochrome *c* peroxidase and lignin peroxidase Compounds II into the ferric state in the absence of electron donor substrates has been explained by the presence of numerous aromatic residues in their structure (3). Unlike fungal and microbial peroxidases, classic plant peroxidases contain only a single highly conserved Trp residue [Fig. 1, adapted from the review (4, 5)] which is located between two alpha-helices at the side opposite to the entrance to the heme-binding pocket (Fig. 2), ca. 8–9 Å apart from the heme edge. This highly conserved Trp probably plays an important structural role, however, it might be also indirectly involved in peroxidase catalysis. The detection of the enzyme Trp-fluorescence in the course of tyrosine oxidation catalyzed by HRP (6) and the recent observation of a protein-bound radical in His42Leu mutant HRP after its rapid mixing with hydrogen peroxide (Profes-

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Abbreviations used: HRP, horseradish peroxidase, TOP, tobacco anionic peroxidase, PNP, peanut cationic peroxidase, SBP, soybean peroxidase, ABTS, ammonium 2,2′-azino-bis-(3-ethylbenzothiasoline-6-sulfonate).

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HRPC   1: QLTPTFYDNSCPNVSNIVRDTIVNELRSDPRIAASILRLHFHDCFVNGCD  50
PNP    1: QLSSNFYATKCPNALSTIKSAVNSAVAKEARMGASLLRLHFHDCFVQGCD  50
TOP    1: QLSATFYDTTCPNVTSIVRGVMDQRQRTDARAGAKIIRLHFHDCFVNGCD  50
SBP    1: QLDPSFYRDTCPRVHSIVREVVRNVSKKDPRMLASLIRLHFHDCFVQGCD  50

HRPC  51: ASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVESACPRTVSCADL 100
PNP   51: ASVLLDDTSNFTGEKTAGPNANSIRGFEVIDTIKSQVESLCPGVVSCADI 100
TOP   51: GSILLDTDGT-QTEKDA-PANVGAGGFDIVDDIKTALENVCPGVVSCADI  98
SBP   51: ASVLLNNTATIESEQQALPNNNSLRGLDVVNYIKTAVEKACPGVVSCADI 100

HRPC 101: LTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLDLANANLPAPFFTLPQLKD 150
PNP  101: LAVAARDSVVALGGASWNVLLGRRDSTTASLSSANSDLPAPFFNLSGLIS 150
TOP   99: LALASEIGVVLAKGPSWQVLFGRKDSLTANRSGANSDIPSPFETLAVMIP 148
SBP  101: LTLASQISSVLGGGPHWKVPLGRRDSLTANRNLANQNLPAPFFNLSRLKA 150

HRPC 151: SFRNVGLNRSSDLVALSGGHTFGKNQCRFIMDRLYNFSNTGLPDPTLNTT 200
PNP  151: AFSNKGFT-TKELVTLSGAHTIGQAQCTAFRTRIYNESN---IDP----T 192
TOP  149: QFTNKGMD-LTDLVALSGAHTFGRARCGTFEQRLFNFNGSGNPDLTVDAT 197
SBP  151: AFAVQGLD-TTDLVALSGAHTFGRAHCNFILDRLYNFSGTGKPDPTLDTT 199

HRPC 201: YLQTLRGLCPLNGNLS-ALVDFDLRTPTIFDNKYYVNLEEQKGLIQSDQE 249
PNP  193: YAKSLQANCPSVGGDT-NLSPFDVTTPNKFDNAYYINLRNKKGLLHSDQQ 241
TOP  198: FLQTLQGICPQGGNNGNTFTNLDISTPNDFDNDYFTNLQSNQGLLQTDQE 247
SBP  200: YLQQLRQICPNGGPN--NLVNFDPVTPDKIDRVYFSNLQVKKGLLQSDQE 248

HRPC 250: LFSSPNATDTIPLVRSFANSTQTFFNAFVEAMDRMGNITPLTGTQGQIRL 299
PNP  242: LFNGV---STDSQVTAYSNNAATFNTDFGNAMIKMGNLSPLTGTSGQIRT 288
TOP  248: LFST-SGSATIAIVNRYAGSQTQFFDDFVSSMIKLGNISPLTGTNGQIRT 296
SBP  249: LFSTP-GADTIPIVNRFSSDQKVFFDAFEASMIKMGNIGVLTGKKGEIRK 298

HRPC 300: NCRVVNSNS                                        308
PNP  289: NCRKTN                                           294
TOP  297: DCKRVN                                           302
SBP  299: HCNFVN                                           302
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FIG. 1. Alignment of amino acid sequences of plant peroxidases from horseradish (HRP), peanut (PNP), tobacco (TOP), and soybean (SBP).

sor R. N. F. Thorneley, personal communication) provide some evidence for the involvement of Trp-117 in peroxidase catalysis.

To test the role of the single Trp residue in HRP we have constructed the mutant Trp117Phe. The data show that this Trp plays a key role in protein folding, but not in the protein stability.

MATERIALS AND METHODS

Materials. 2,2′-Azino-bis(3-ethyl-thiasoline-6-sulfonate) diammonium salt (ABTS), *o*-phenylene diamine, luminol, *p*-iodophenol were from Sigma. All chemicals used for the genetic engineering manipulations were of "Molecular Biology Grade" (Sigma). Plasmid pSA261 with the HRP gene under tac-promoter control was kindly provided by Amersham International plc (UK).

Preparation of recombinant wild-type and mutant Trp117Phe horseradish peroxidase. Wild-type recombinant horseradish peroxidase was expressed in *E. coli JM109/pSA261* and refolded from inclusion bodies as previously described (7). Single-point mutagenesis was performed using the synthetic horseradish peroxidase gene (Amersham International plc, UK) and the primer (5′-cc-taa-tgg-tac-tcg-gaa-tga-ggg-ccc-gcc-g-3′) providing the replacement of Trp-117 with Phe. The mutagenesis was performed by the same protocol as described previously for Phe41His and Phe143Glu single-point mutants (7). Both strands of the final plasmid construction were sequenced on a Applied Biosystems Automated Model 370 DNA Sequencer (Perkin–Elmer–ABI) using ABI PRIZM DNA sequencing kit. The mutant was refolded from *E. coli* inclusion bodies using our published protocol (7) with the following modifications. The optimal composition of refolding medium was achieved varying pH over the range 8.0–10.0 and adding ammonium sulfate (up to 20% of saturation).

pH stability of mutant and wild-type recombinant HRP (10^{-7} M solutions) was studied over the range of pH 3–10 in 10 mM citrate/phosphate buffer solution. Irradiation was performed using a γ-source with the dose power of $P_\gamma = 0.05$ Gy/s. Enzyme solution (0.1 μM in 50 mM Tris–HCl buffer, pH 7.0) was placed into Eppendorf tubes and the retained enzymatic activity toward ABTS and *o*-phenylene diamine was measured 1 h after the cessation of irradiation (incubation at 20°C).

Steady-state kinetics experiments. ABTS oxidation was studied in 0.1 M citrate/phosphate buffer, pH 4.8, at 405 nm using a molar absorptivity of 36.8 mM^{-1} cm^{-1} (8). ABTS and H_2O_2 concentrations were varied over the ranges 15.6–500 and 8–400 μM, respectively. Concentrations of the enzymes were 0.17 nM for the native and recHRP and 0.16 nM for the mutant.

o-Phenylene diamine oxidation was studied in 0.1 M citrate/phosphate buffer, pH 4.8, at 450 nm using a molar absorptivity of 11.1 mM^{-1} cm^{-1} (9). *o*-Phenylene diamine and H_2O_2 concentrations were varied over the ranges 7–220 and 8–400 μM. Concentrations of the enzymes were 0.21 nM for native and recHRP and 0.56 nM for the mutant.

Chemiluminescence measurements were made on a 1251-002 Luminometer (Wallac). Sample volume was usually 1 ml. The reactions were initiated by simultaneously dispensing substrate solution. The optimized luminescent reagent contained 1 mM luminol, 10 mM *p*-iodophenol and 1.8 mM H_2O_2 in 0.2 M Na–borate buffer, pH 8.5. Concentrations of the enzymes were 1.96, 1.27, and 2.0 pM for the native, recombinant wild-type and mutant Trp117Phe HRP, respectively.

RESULTS AND DISCUSSION

The first difference between the wild-type recombinant enzyme and Trp117Phe mutant was observed at the step of protein refolding from inclusion bodies sol-

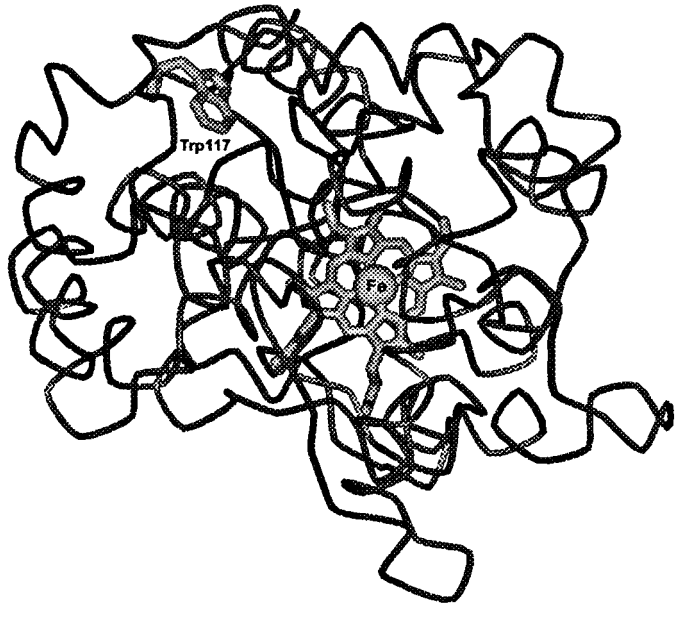


FIG. 2. Crystal structure of horseradish peroxidase shown from the distal site of the molecule. The replaced Trp-117 is ca. 8.9 Å from the heme edge.

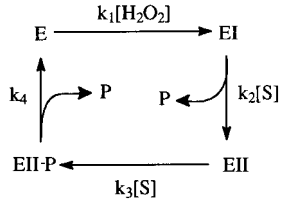
TABLE 1

Biochemical and Kinetic Characteristics of HRP Forms

	Native HRP	wt-recHRP	W117F recHRP
Molecular mass, kDa	44	34	34
Isoelectric point, p*I*	8.90	9.05	9.05
Specific activity with ABTS, U/mg	1500	2000	1200
Rate constants with ABTS			
k_1, $M^{-1}\ s^{-1} * 10^{-7}$	1.2	1.2	1
k_3, $M^{-1}\ s^{-1} * 10^{-6}$	2	2.5	3
k_4, $s^{-1} * 10^{-5}$	—	26	2.5
Rate constants with *o*-phenylene diamine			
k_1, $M^{-1}\ s^{-1} * 10^{-7}$	1.0	0.5	0.8
k_3, $M^{-1}\ s^{-1} * 10^{-7}$	3	3	3.5
k_4, $s^{-1} * 10^{-6}$	—	3	3.7

ubilized in 6 M urea. The yield of the wild-type enzyme under the optimized refolding conditions (20 mg protein per liter, pH 9.3) was ca. 20,000–25,000 U per liter of refolding medium as we wrote earlier (7) while the yield of Trp117Phe under the same refolding conditions was only 1500 U per liter. The pH-optimum for the mutant refolding was shifted to pH 8.5, and the addition of ammonium sulfate (20% of saturation) was essential for optimum yields of active enzyme. We speculate that ammonium sulfate stimulates hydrophobic interactions within the enzyme molecule and thus, helps with the enzyme refolding in the absence of the single Trp residue. This newly optimized refolding procedure for Trp117Phe mutant allowed the yield of 8000 U/liter to be achieved. The purification procedure was the same as for the wild-type enzyme, and the specific activity of the mutant with ABTS as a substrate was ca. 1100–1500 U/mg that was comparable with the wild-type enzyme (2,000 U/mg). The biochemical and kinetic properties of Trp117Phe mutant are presented in Table 1.

The characteristic feature of the recombinant HRP and its variants is the appearance of a new rate-limiting step (k_4) that is usually assigned to the product dissociation (10). Native HRP and Trp117Phe variant have similar rate constants for *o*-phenylene diamine oxidation (Table 1). The kinetics of ABTS oxidation by Trp117Phe shows that for this substrate k_4 drops by an order of magnitude. The assignment of k_4 to product dissociation was rationalized for recombinant HRP by the more hydrophobic nature of recombinant enzyme due to the absence of glycans. However, it is difficult to imagine that the introduction of a single-point mutation replacing one aromatic residue with another could result in such a dramatic increase in enzyme hydrophobicity to induce an order of magnitude decrease in the rate constant for ABTS cation-radical dissociation. The kinetic scheme used by A. T. Smith and coauthors (10) (Scheme 1) is the simplest one that corresponds to the experimentally obtained rate equation $2E_o/v = 1/k_4 + 1/k_3[\text{ABTS}] + 1/k_1[H_2O_2]$. If we take into account the formation of Michaelis complexes between peroxidase Compound I/II and a reducing substrate yielding the corresponding enzyme-product complexes (Schemes 2 and 3) the rate equations will look the same with k_4 representing a combination of elementary rate constants:

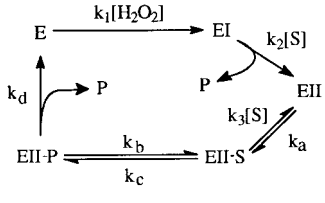


SCHEME 1

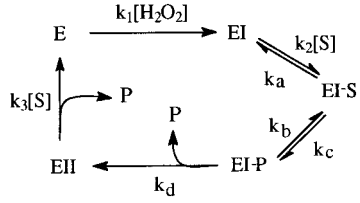
$$1/k_4 = (k_c + k_d + k_b)/k_b \cdot k_d. \qquad [4]$$

The observed rate-limiting step could correspond either to electron transfer in Michaelis complex or product dissociation. If the product dissociation step is not rate-limiting, i.e., $k_d \gg k_b > k_c$, the rate-limiting step could correspond to electron transfer through the peroxidase molecule ($1/k_4 = 1/k_b$). Thus, the kinetic data obtained in this study can be rationalized assuming for the participation of Trp-117 in electron transfer pathways through HRP.

The enzyme stability was investigated using different methods. The mutant enzyme exhibited a higher stability in acid media (pH < 5) compared to the wild-type enzyme (not shown). Inactivation during the reaction course was studied using peroxidase catalyzed cooxidation of luminol and *p*-iodophenol (the so-called enhanced chemiluminescence). The decay of the chemiluminescent signal occurs due to the enzyme inactivation by phenoxy radicals generated from the oxidized enhancer-substrate (*p*-iodophenol). The mutant enzyme was more stable consistent with Trp acting as a radical trap (Fig. 3). However, the stabilization effect of the mutation was still insufficient compared to the glycosylated native enzyme where oligosaccharide chains play a protective role (11).



SCHEME 2



SCHEME 3

The most interesting results were obtained using the method of radiation-induced inactivation developed by us earlier to characterize structural and conformational changes in recombinant HRP forms (12). Irradiation of HRP solutions results in formation of Compound II which slowly converts into ferric enzyme on cessation of irradiation (13). Proteins are thought to be inactivated mostly via amino acid residue modification with hydroxyl radicals and solvated electrons generated by water radiolysis. His, Trp, and Tyr are thought to be most reactive. Thus, the removal of Trp residue should stabilize the HRP molecule against radiation-induced inactivation. And this was actually observed (Fig. 4). The inactivation curve for the wild-type enzyme shows an initial drop and then a plateau where the enzyme is comparatively stable (12). For the mutant no initial drop was observed, and so, we can speculate that Trp is the first target modified by radicals produced by water radiolysis and this modification destabilizes the enzyme molecule to some extent. Another feature of the Trp117Phe mutant was the independence of the inactivation curve with respect to the substrate used to measure the enzyme activity (not shown). For the wild-type enzyme, we demonstrated

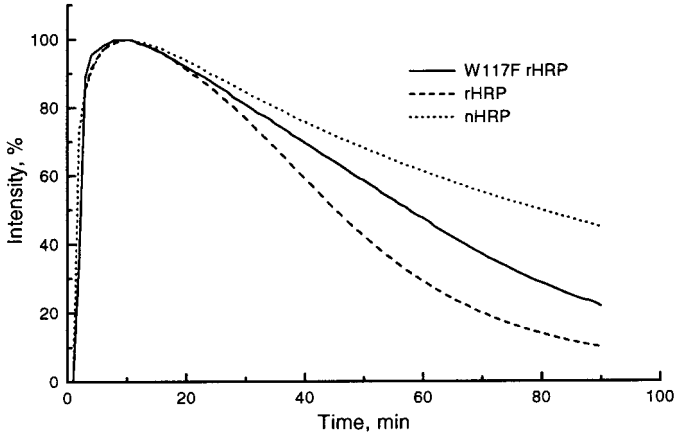


FIG. 3. Time course of chemiluminescent signal for the native (· · ·, nHRP), recombinant wild-type (- - -, rHRP), and mutant Trp117Phe rHRP (—, W117F rHRP). Reaction conditions: 1 mM luminol, 10 mM *p*-iodophenol and 1.8 mM H_2O_2, 0.2 M Na–borate buffer, pH 8.5. Concentrations of the enzymes were 1.96, 1.27, and 2.0 pM for the native, recombinant wild-type, and mutant W117F HRP, respectively.

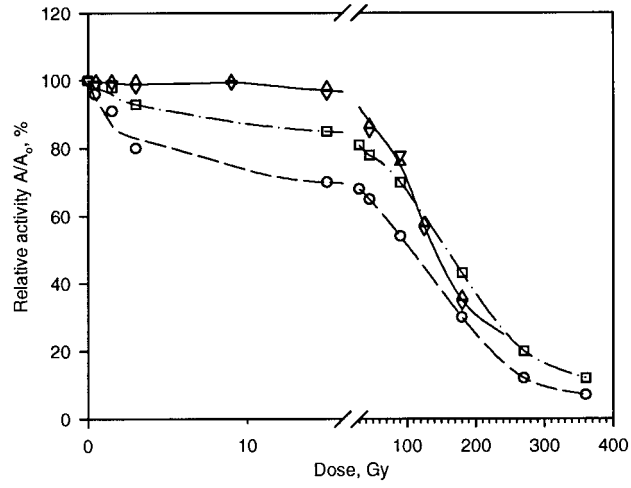


FIG. 4. Radiation-induced inactivation of the wild-type rHRP and Trp117Phe mutant before (□ and △, respectively) and after (○ and ▽, respectively) the enzyme pretreatment with equimolar hydrogen peroxide with ABTS used as a substrate. The enzyme concentration was 0.1 μM (50 mM Tris–HCl buffer, pH 7.0). The initial activity (A_o) was taken as 100%. Irradiation was performed using a γ-source with the dose power of $P_\gamma = 0.05$ Gy/s.

that the activity toward ABTS was less stable than that toward guaiacol and *o*-phenylene diamine (12). This result can be rationalized if one suppose that Trp somehow participates in ABTS oxidation (possibly by forming an extra electron transfer pathway in the enzyme molecule).

The data obtained in this study show that Trp117 play a significant role in protein folding. However, the presence of Trp-117 destabilizes the protein with respect to radical inactivation. If Trp-117 is involved into electron transfer pathways in HRP, its removal will decrease the "molecular conductivity" of HRP, and thus, provide the enzyme stabilization against the attack of hydroxyl radicals and solvated electrons generated by water radiolysis. The kinetic data can be interpreted in terms of Trp-117 participation in electron transfer within the Michaelis complex. Thus, the set of data obtained in this study give indirect evidence for Trp-117 participation in electron transfer pathways through HRP molecule. We believe that the ongoing characterization of the recombinant HRP forms in direct electron transfer reactions on graphite and gold electrodes will allow us to clarify the role of Trp-117 in peroxidase catalysis.

ACKNOWLEDGMENTS

The authors thank Professor Lo Gorton (Lund University, Sweden) for his continuous interest in this work and Professor R. N. F. Thorneley (John Innes Centre, Norwich, UK) for valuable comments on the manuscript. The research was supported by EU-Grants INCO-Copernicus (Contract IC15-CT96-1008) and DIAMONDS (BIO4-CT97-2199).

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