Catalytic properties of Phe41→His mutant of horseradish peroxidase expressed in *E. coli*

*D. B. Loginov, I. G. Gazaryan, V. V. Doseeva, A. G. Galkin, V. I. Tishkov, E. A. Mareeva, and M. A. Orlova**

M. V. Lomonosov Moscow State University, Department of Chemistry, Leninskie Gory, 119899 Moscow, Russian Federation. Fax: +7 (095) 939 0126

The recombinant horseradish peroxidase and its single-point F41H mutant have been reactivated from *E. coli* inclusion bodies. The influence of the mutation on the heme entrapment, stability and activity of the enzyme was demonstrated. The catalytic rate constants for H_2O_2 cleavage and ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) oxidation decrease by two and one orders of magnitude, respectively. Unlike the wild-type recombinant horseradish peroxidase, the elimination of the ABTS oxidation product is not a rate-determining step for the mutant. The F41H replacement results in significant changes of kinetics of iodide ion oxidation. The reaction rate is linear to the concentrations of iodide, H_2O_2, and the enzyme. The results suggest the direct interaction of iodide with the porphyrin ring of the heme. The decrease in ABTS oxidation activity accompanied by retention of activity in iodide oxidation in the course of low-dosage radiolysis of the F41H mutant is additional evidence of the direct electron transfer from iodide to the heme, in contrast to ABTS oxidation, in which the electron transfer chain in the protein molecule is involved.

Key words: recombinant horseradish peroxidase, mutagenesis, kinetics of enzymatic oxidation, radiation inactivation.

Horseradish peroxidase (HRP) is a heme-containing peroxidase, as is yeast cytochrome—*C*—peroxidase, for which the crystal structures of both native protein[1] and its mutant variants[2] are known. HRP is the most extensively studied plant peroxidase. However, the data on its atomic structure are absent. The success achieved in expression of the gene of HRP in the systems of *E.coli*[3,4] and baculovirus[5] expression allows one to use protein engineering methods to study this enzyme. The basic direction in these studies is connected with elucidation of peculiarities of its catalytic mechanism, identification of the amino acid residues directly involved in heterolytic cleavage of H_2O_2, and subsequent electron transfer from the donor substrate to the enzyme. On the basis of the known data on protein engineering of cytochrome—*C*—peroxidase and the data on studies of heme-containing peroxidase by X-ray analysis, NMR, Raman, and Mössbauer spectroscopic methods, one can assume that the first stage of the catalytic process, *i.e.*, heterolytic cleavage of H_2O_2, yields so-called compound I of peroxidase, which is an oxyferryl-enzyme, containing the radical either at the Trp-191 residue in cytochrome *c* peroxidase or at the porphyrin ring of HRP.

The two histidine residues of HRP, *i.e.*, the proximal His-170 and the distal His-42 ones (residues 175 and 52 in cytochrome—*C*—peroxidase, respectively) and their immediate environment play a key role in this transformation. The exact role of the Phe-41 residue, which neighbors the distal histidine, thus far remains unclear.

To specify the role of distal Phe-41, we obtained and investigated for the first time the HRP mutant, in which this residue was replaced with histidine (F41H mutant). Previously, mutant forms with valine (F41V) and tryptophan (F41W) instead of Phe-41 have been prepared and characterized.[6] Participation of the protein in the second stage of the catalytic process (the transfer of the two oxidation equivalents from the enzyme to the donor substrate) was not practically studied. Only some assumptions on the difference in location of the binding centers for iodide, guaiacol, and thioanisol are given[7], along with NMR data on participation of the phenylalanine residues (probably, Phe-142 and Phe-143) in binding of aromatic donors.[8]

Results and Discussion

The procedure for the preparation of recombinant horseradish peroxidase (RHRP) from *E.coli* inclusion bodies previously developed by us[4] and improved further allows one to obtain *ca.* 25 mg of the recombinant enzyme from 1 L of culture possessing specific activity *ca.* 4000 E per 1 mg of the protein.[9] One of peculiarities of this procedure[9], unlike the one previously reported[4] is

Translated from *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 11, pp. 2034—2038, November, 1994.

1066-5285/94/4311-1923 $12.50

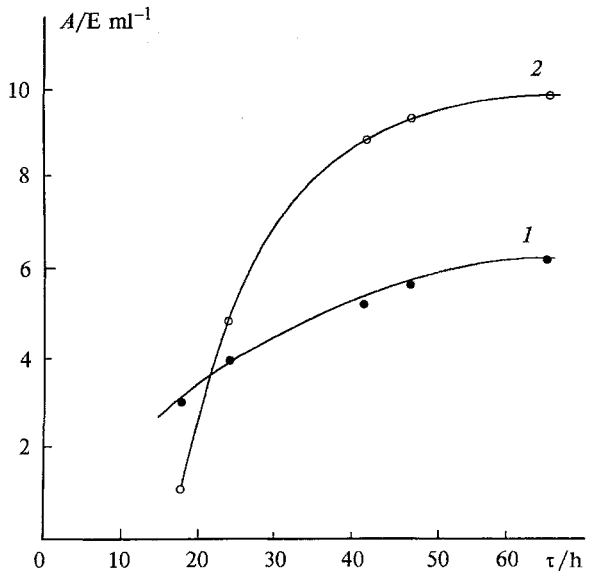

Fig. 1. Effect of the order of hemin addition to reactivation medium on the yield of RHRP: after completion of formation of the active apoenzyme (*1*), and in the process formation of the active apoenzyme (*2*). The concentration of the protein is 10 mg L^{-1}.

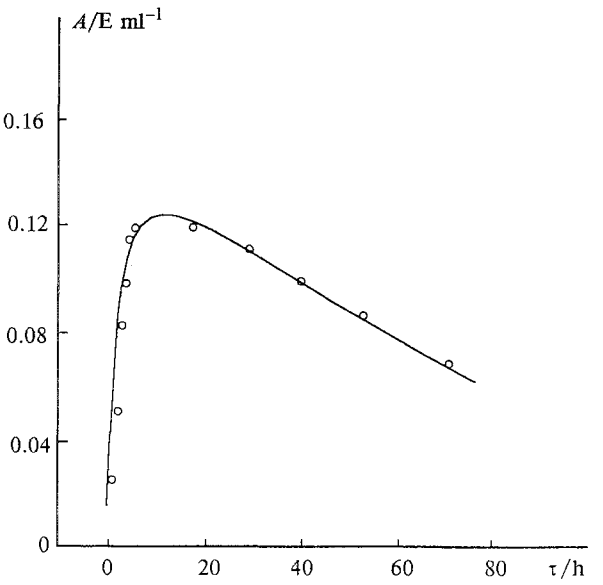

Fig. 2. Kinetics of hemin entrapment in the RHRP-F41H mutant after formation of the active apoenzyme. The concentration of the protein is 10 mg mL^{-1}.

the addition of hemin to the medium after reactivation of the apoform of RHRP. In this case, the yield of activity practically doubles (Fig. 1, curve *1*) in comparison with the direct reactivation of the holoform (Fig. 1, curve *2*). This observation is in accordance with the previously obtained data on renaturation of native HRP in the presence of guanidinium chloride, and the proposed mechanism of denaturation—renaturation of the enzyme,[10] in which hemin is not a primer in the process of folding of the protein globule and therefore can be added after renaturation of the apoenzyme. The much higher yield of the active enzyme in the case of addition of hemin in our procedure can be explained by the ability of hemin to catalyze oxidation of the SH group with atmospheric oxygen and thus to accelerate the formation of S—S bonds, including erroneous.

The kinetics of entrapment of heme in RHRP apoenzyme (Fig. 1, curve *1*) indicates that the process of formation of the holoenzyme is completed after 40—42 h of incubation. The kinetics of hemin entrapment into RHRP-F41H and RHRP of wild type are not the same (Fig. 2). Hemin rapidly is included into RHRP-F41H; the maximum yield of activity is achieved after 5 h of incubation, then irreversible inactivation of the enzyme begins, apparently due to instability of the mutant form in the basic medium. Thus, even at this stage, alterations in the properties of the mutant obtained become evident. Among them, in particular, are its lowered stability (Table 1) and the decrease in rate of heme entrapment, which may be connected with changes in the structure of the heme-binding pocket due to the appearance of the His residues.

The yield of mutant form decreases three-fold in comparison with the wild-type RHRP. An attempt to apply methods of immunoaffinity chromatography for the purification of the mutant form leads to practically complete inactivation of the enzyme (yield is less than 10 %).

Taking into account the low specific activity of the mutant form of the enzyme, one can suggest the significant influence of this point mutation upon the RHRP activity. The mutant was practically inactive with respect to guaiacol also in the so-called phenol-antipyrine assay. The level of activity with respect to other substrates was 1—2 % that of wild-type RHRP. In order to esti-

Table 1. Preparation and purification of RHRP-F41H mutant

Stage of purification*	Total protein / mg	Total activity *E*	Specific activity *E*/mg	Yield** (%)	Reactivation of protein (%)
		Wild-type RHRP[9]			
1	20	0	0	0	
2	20	20000	1000	100	
3	20	24000	1200	120	
4	5	20000	4000	100	25
		Mutant RHRP-F41H			
1	20	0	0	0	
2	20	240	12	100	
3	20	270	3.5	112	
4	1.5	145	90	60	7.5

* 1, solubilization of RHRP apoprotein in 6 *M* urea; 2, reactivation; 3, precipitation with ammonium sulfate; 4, gel filtration. ** Yield of activity.

mate the measure of mutation effect on every stage of peroxidase catalysis, the oxidation of ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and iodide anion by the methods of stationary kinetics over a wide range of concentrations of both substrates was investigated.

The kinetic data on ABTS oxidation presented in the double reciprocal coordinates ($1/v \div 1/[S]$) are presented as a family of parrallel straight lines described by the equation:

$$2[E_0]/v = 1/k_1[H_2O_2] + 1/k_3[ABTS]^6,$$

where k_1 and k_3 are the apparent rate constants of H_2O_2 cleavage and oxidation of ABTS. The monomolecular rate-determining stage of the reaction (k_4),

$$E + H_2O_2 \longrightarrow EI + H_2O \quad (k_1),$$

$$EI + ABTS \longrightarrow EII + P \quad (k_2),$$

$$EII + ABTS \longrightarrow E\text{-}P \quad (k_3),$$

$$E - P \longrightarrow E + P \quad (k_4),$$

which is characteristic for wild-type RPH and connected with dissociation of ABTS oxidation product[6,9] was absent for the mutant form. In accordance with the data from Table 2, the F41H mutation causes a decrease in the catalytic rate constants by two orders of magnitude. However, the alterations at the stage of heterolytic cleavage of H_2O_2 are more profound: k_1 is 200 times less, whereas k_3 is 15 times less than the corresponding values for the recombinant wild-type enzyme. In the case of F41V mutant, described by Smith[6], the mutation causes a decrease in the catalytic constant of the H_2O_2 cleavage by one order of magnitude (Table 2), *i.e.*, the mutation predominantly effects the first stage of catalysis.

The data obtained confirm the important role of the Phe-41 residue localized between the His-40 and His-42 residues in the mechanism of catalysis with HRP. His-42 coordinates the iron atom of hemin in the HRP active site. Thus, the F41H mutation leads to the appearance of a sequence with three His residues in the active site of HRP. This arrangement should assist in binding of hemin with the protein. In fact, the fast formation of the holoform from the apoform after addition of hemin took place, but also destabilization of the enzyme and decrease in its catalytic activity were observed. Previously, it was shown that replacement of Phe-41 by tryptophan and valine residues[6] did not cause a significant decrease of enzymatic activity (specific activity decreased by 3—8 times). It should be noted that both tryptophan and valine, as well as substituted phenylalanine, are hydrophobic amino acid residues. Imidazole of histidine is an ionogene in basic solutions. The F41H replacement results in a decrease in catalytic activity by 44 times, thus affecting both steps of the H_2O_2 cleavage and ABTS oxidation. The probable result of the replacement of phenylalanine by histidine is the destruction of the hydrophobic heme-binding pocket and the increase in flexibility of the heme inside the active site. The study of properties of the F41H mutant allows one to consider Phe-41 as a hydrophobic barrier separating the two distal histidine residues and providing strong non-covalent interaction of the protein with the rather hydrophobic porphyrin ring of hemin.

Table 2. Apparent rate constants of ABTS oxidation with H_2O_2, catalysed by recombinant forms of HRP (0.1 *M* Na-acetate buffer, pH 5.0, 25 °C). Relative error does not exceed 10 %

Catalyst	$k_1/\mu M^{-1}\ s^{-1}$	$k_3/\mu M^{-1}\ s^{-1}$	k_4/s^{-1}
RHRP	4.8	3.0	4600
F41H	0.022	0.37	—
RHRP[6]	5.9*	3.7	850
F41V[6]	0.63	0.81	90

* Experimental error *ca.* 50 %.

Unlike ABTS, the iodide anion is a two-electron substrate, and the oxidation proceeds in one stage. As reported by Ortiz de Montellano,[7] iodide binds near the δ-meso-ring of hemin. The kinetic mechanism of oxidation of the iodide anion, catalysed by the wild-type RHRP, obeys the Michaelis —Menten equation, and is described by the following equation:

$$[E_0]/v = 1/k_1[H_2O_2] + 1/k_2[KI],$$

where k_1 and k_2 are the apparent rate constants of the H_2O_2 cleavage and the oxidation of iodide, which are 0.8 $\mu mol^{-1}\ L\ s^{-1}$ and 7 $mmol^{-1}\ L\ s^{-1}$, respectively. In the case of the HRP mutant, the linear correlation of the reaction rate with the concentration of the substrates and the enzyme, *i.e.*, the rate equation is as follows:

$$v = k[E_0][H_2O_2][KI],$$

where k is the third order rate constant, equal to $2.5 \cdot 10^5\ mol^{-2}\ L^2\ s^{-1}$. The data on kinetics of oxidation of iodide suggest that the iodide ion reacts directly with π-radical cation of porphyrin without participation of the protein component.

To verify this assumption, the method of radiation-induced inactivation was used, which was applied previously for comparison of the substrate specificity of native and recombinant enzymes[9]. Radiolysis of the RHRP-F41H solutions (Fig. 3) results in a fast decrease in activity for ABTS, while the activity for iodide remains unchanged for a long time.

Radiation-induced inactivation of HRP proceeds through damage of the protein globule by interaction with radicals generated in a solution. Modification of the amino acid residues probably results in conformational changes, damaging both the whole structure of the molecule and the binding site of the substrate (the electron transfer chain from the substrate to the active center, *i.e.*, heme). At the final stages of inactivation the destruction of the secondary structure takes place and

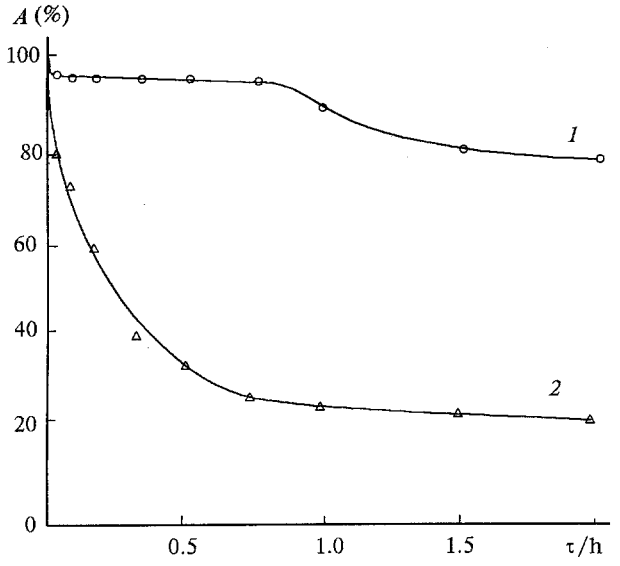

Fig. 3. Change in activity with respect to KI (*1*) and to ABTS (*2*) in the process of radiation-induced inactivation of the RHRP-F41H mutant. P_γ = 0.05 Gy s^{-1}.

«leakage» of hemin into the solution proceeds. Dissociation of the non-covalent bonds of heme with the protein molecule proceeds in the presence of guanidinium chloride, as was shown by studies of inactivation,[10] only at the stage of destruction of the secondary structure, *i.e.*, at the final step.

The stability of the RHRP-F41H activity with respect to iodide indicates that this activity remains constant until radiation-induced inactivation results in total destruction of the secondary structure with «leakage» of heme into the solution, *i.e.*, just the retention of heme coordination with the protein is sufficient for manifestation of this activity. To put it otherwise, the electron transfer from porphyrin to iodide proceeds without participation of the protein component. In the case of ABTS, the corresponding structural organization of the binding site is necessary for the exhibition of the activity. Evidently, in the case of oxidation of ABTS, the protein organizes the chain of electron transfer, but in oxidation of the iodide ion the substrate is oxidized directly by the π-radical cation of hemin.

Finally, some conclusions can be made. First, the point mutation F41H significantly affects the structure of the active site of the enzyme, thus suggesting that the main role of Phe-41 is the provision of the right structure of the hydrophobic pocket for the non-covalent binding of hemin. Second, the change in mechanism of oxidation of iodide and the character of the change in activity under radiolysis indicates the direct contact of the iodide ion with the porphyrin ring of heme in the process of two-electron transfer. Therefore, in the case of oxidation of iodide the role of the protein environment consist only of ensuring heterolytic H_2O_2 cleavage, unlike in the case of oxidation of ABTS, for which the formation of the electron transfer chain by the protein is necessary.

Experimental

Reagents: Ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), isopropyl-β-D-thiogalactopyranoside (IPTG), Na dodecylsulfate (SDS), tris-hydroxymethylaminomethane (TRIS), oxidized glutathione, dithiothreitol (DTT), hemin (Sigma, USA); bactotripton and yeast extract (Difco); restrictases, ligases and other enzymes for mutagenesis, including a Sculptor™ IVM System kit for directed mutagenesis and the original plasmide pSA261 with the HRP gene under control of *tac*-promotor were kindly supplied by Amersham International plc (UK).

Methods. The measurements of peroxidase activity were performed with a Shimadzu UV 120-02 spectrophotometer (Japan) at 25 °C: ABTS: 0.05 mL of ABTS solution (15 mmol L^{-1}) and the enzyme aliquot were added to 2 mL of 0.1 mol L^{-1} Na-acetate buffer, pH 5.0; the reaction was initiated by addition of 0.1 mL of 0.5 % solution of H_2O_2 (ε_{405} = 36800 mol L^{-1} cm^{-1}).[11] Activity was presented in *E* units (μmol min^{-1}) per 1 mg of protein.

Apparent rate constants of the oxidation reactions of ABTS and KI for RHRP were determined from the data on stationary kinetics, varying the concentration of one of substrates while the concentrations of the second substrate were kept constant. For wild-type RHRP concentrations of the substrates were varied in ranges: 0.015—0.15 mmol L^{-1} of ABTS, 0.5—5 mmol L^{-1} of KI, 0.010—1 mmol L^{-1} of H_2O_2, 1—10 nmol L^{-1} of the enzyme, for mutant forms those are 0.015—0.15 mmol L^{-1} of ABTS, 0.5—5 mmol L^{-1} of KI, 0.5—5 mmol L^{-1} of H_2O_2, 10—80 nmol L^{-1} of the enzyme. In the case of oxidation of KI an increase of absorption was monitored at 350 nm, assuming ε_{350} = 26000 mol L^{-1} cm^{-1}.[12] The concentration of H_2O_2 was determined using the millimolar extintion coefficient (43.6 mol L^{-1} cm^{-1}),[13] and ABTS and KI were determined by weight.

RHRP was prepared as described previously[9]. RHRP-F41H was obtained by a method of directed mutagenesis of one-chain DNA. To replace the sequence CAT(His-40)-TTT(Phe-41)-CAT(His-42)-GAC(Asp-43) to CAT(His-40)-CAC(His-41)-CAT(His-42)-GAC(Asp-43) one 29-oligomer 5′-CCTT-CGT-CTA-CAT-CAC-CAT-GAC-TGC-TTTG-3′ was synthesized with an Applied Biosystems 380B synthesizer. The selection of mutant DNA was based on disappearance of the restriction site BspHI (TCATGA). The final constructions were obtained with an Applied Biosystems 370A sequenator using Taq-polymerase and dye-labelled primers.

Synthesis, purification, and characterization of the mutant forms of RHRP were performed using a procedure similar to that used for preparation of wild-type RHRP.[9] Irradiation of the aqueous solution (10^{-7} mol L^{-1}) of HRP mutant was performed on γ-source (P_γ = 0.05 Gy s^{-1}). Then the enzymatic activity was measured for different substrates, considering spontaneous and post-irradiation inactivation.

Protein content was determined spectrophotometrically.[14] Homogeneity of the enzyme preparations was tested by electrophoresis in polyacrylamide gel in the presence of SDS.

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Received September 27, 1994