Effect of a negative charge on the screening of the active site of horseradish peroxidase

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The F143E mutant form of the recombinant horseradish peroxidase was reactivated from *E. coli* inclusion bodies. The mutation inhibits heme entrapment and results in a decrease in the catalytic activity, mainly affecting the stage of the oxidation of a donor substrate (ABTS, iodide). An increase in stability of the mutant form obtained under radiation inactivation over that of the wild-type recombinant enzyme was observed. The data obtained confirms the proposed location of Phe143 at the entrance of the active center, hence its replacement by the negatively charged glutamic acid residue retards heme entrapment and substrate binding, thus protecting the active center of the enzyme against the radicals generated by radiolysis.

Key words: recombinant horseradish peroxidase, mutagenesis, radiation inactivation, F143E.

The homology of the amino acid sequences of heme-containing peroxidases of different types, in which hemin is coordinated in the active center, is very high (100 % in the distal area and *ca.* 80 % in the proximal area). The activity towards different substrates varies over a wide range. Thus, alfalfa peroxidase possesses high activity towards guaiacol, horseradish peroxidase (HRP) is highly active towards ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and tobacco peroxidase is highly active towards luminol. Meanwhile, all plant peroxidases are inactive towards veratrole, the model substrate of lignin-peroxidase. Apparently, the chain of electron transfer from the donor substrate to the oxidized active center (oxyferryl-heme) exhibits particular features in every peroxidase. It became possible to study the molecular determinants of the substrate specificity of heme-containing peroxidases due to cloning and gene expression in heterologous systems of *E. coli*, *Aspergillus,* and insect cell culture[1,2] and also due to the successful determination of the crystal structures of cytochrome *c* peroxidase,[3] E5 isoenzyme of horseradish peroxidase,[4] lignin-peroxidase,[5] and Mn-peroxidase (T. Poulos, unpublished data). A comparison of the amino acid sequences of peroxidases using the structural data[6] allowed one to consider that the entrance to the active center of HRP is formed by the 66-DAFGNAN-72 and 138-LPAPFF-143 sequences. In the latter, Phe142 and Phe143 bind the aromatic substrates, according to the NMR data[7]. Modeling the active center of HRP on the basis of the crystal structure of lignin-peroxidase[4] (Fig. 1) demonstrates that these residues are located close to the *v-meso*-cycle of hemin, which is attached to the binding sites of the substrates, *i.e.*, an iodide ion and guaiacol.[8] Unlike HRP, tobacco peroxidase contains the Glu143 residue. Using the previously developed expression system and reactivation of HRP isolated from *E. coli* inclusion bodies,[6] we have prepared the Phe143→Glu (F143E) mutant. We compared the catalytic properties of the mutant with those of recombinant (RHRP) and native (NHRP) peroxidases

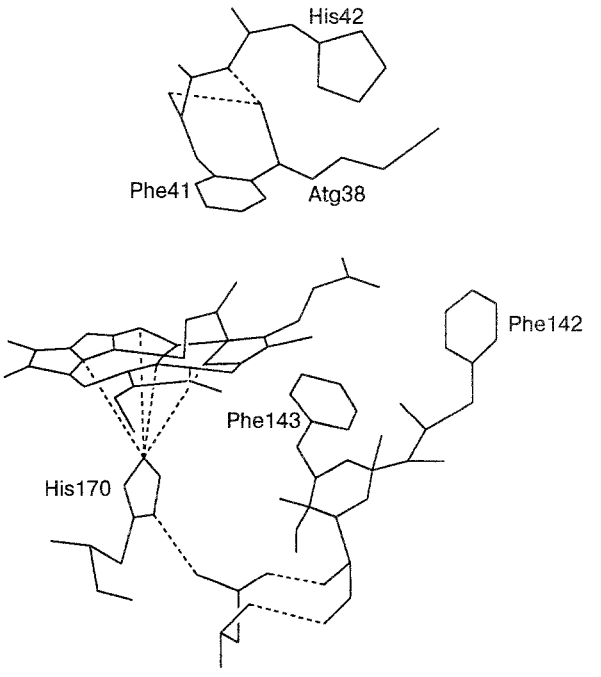

Fig. 1. A model of the active center of horseradish peroxidase.

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Table 1. Preparation and purification of the recombinant forms of HRP

Stage of purification	Total protein /mg	Total activity, *E*/mg	Specific activity, *E*/mg	Yield of act. (%)	Reactivation of protein (%)
		Wild-type RHRP			
1	20	0	0	0	
2	20	20 000	1 000	100	
3	20	24 000	1 200	120	
4	5	20 000	4 000	100	25
		RHRP-F143E			
1	20	0	0	0	
2	20	600	30	100	
3	10	300	30	50	
4	5	250	50	25	25

Note. 1, Solubilization of the apo-protein of HRP in 6M urea, *2,* reactivation, *3,* co-precipitation with ammonium sulfate, *4,* gel filtration.

and tobacco peroxidase in order to elucidate how the Phe residue and its replacement by the Glu residue affect substrate specificity of the enzyme. To consider the mechanism of the effect of the radical on the active center of the enzyme, the method of radiation inactivation, making it possible in some cases to simulate the method of inactivation in the course of the reaction,[9] was used.

Results and Discussion

The data on the reactivation of the mutant form of HRP are presented in Table 1. Along with wild-type RHRP, the procedure developed allowed us to obtain *ca.* 25 mg of the enzyme from *E. coli* inclusion bodies.[10] However, its specific activity appeared low. One of the possible reasons for this fact is the incomplete inclusion of hemin due to a decrease in the rate of the process (Fig. 2). The heme content in the protein-homogeneous preparation of the F143E mutant was only 45 %, in contrast to those of other recombinant forms (98—100 %), which confirms the influence of the mutation on hemin entrapment in the apoenzyme. The F143E mutation results in a significant decrease in activity towards ABTS, and the methods of stationary kinetics were used to elucidate its influence on all of the stages of the catalytic mechanism.

The kinetic data presented in double reciprocal coordinates (1/V to 1/[S]) have the appearance of parallel straight lines described in each case by the equation:[6]

$$2[E_0]/v = 1/k_1[H_2O_2] + 1/k_3[\text{ABTS}] + 1/k_4,$$

where k_1 and k_3 are the apparent rate constants of H_2O_2 cleavage and ABTS oxidation, and k_4 characterizes the rate-determining stage, which can be the stage of product dissociation,[6] and v is the rate of the enzymatic reaction:

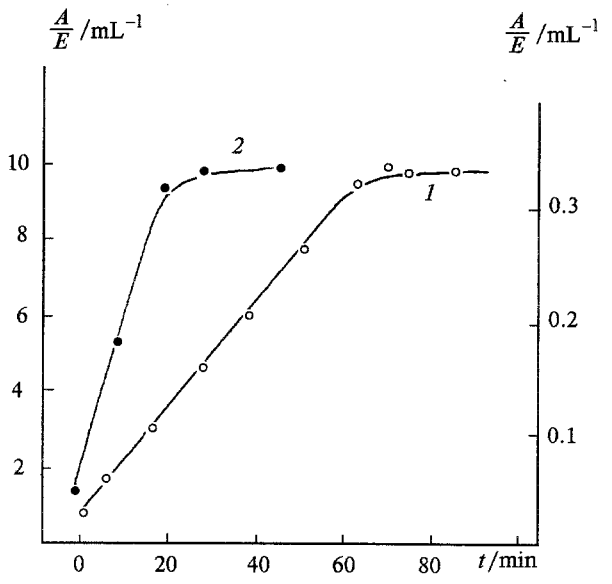

Fig. 2. The kinetics of hemin entrapment in RHRP-F143E (*1*) and in RHRP (*2*). The protein concentration is 10 mg mL^{-1}.

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

$$EI + ABTS \rightarrow EII + P \quad (k_2)$$

$$EII + ABTS \rightarrow E{-}P \quad (k_3)$$

$$E{-}P \rightarrow E + P \quad (k_4)$$

In accordance with the kinetic data (Table 2), the rate constants k_1 and k_3 for the RHRP enzyme preparations obtained in the present work and by A. Smith's group[11] essentially coincide if the experimental error is taken into account. The 5-fold increase in the rate constant of product dissociation for the enzyme prepared by us is of great significance. These data correlate with the value of specific activity, which is greater in 5 times that of the preparation described by A. Smith (880 *E* mg^{-1}).[11] Replacement with F143E mainly re-

Table 2. Apparent rate constants of ABTS oxidation with hydrogen peroxide catalyzed by RHRP

Form of enzyme	Rate constant		
	$k_1/\mu M^{-1}\,s^{-1}$	$k_3/\mu M^{-1}\,s^{-1}$	k_4/s^{-1}
RHRP	4.8	3.0	4600
F143E	1.1	0.32	35
RHRP [6]	5.9*	3.7	850

Note. Conditions: 0.1 *M* Na-acetate buffer, pH 5.0, 25 °C. Error does not exceed 10 % from the value determined.
* Error 50 %.

Table 3. Apparent rate constants of oxidation of the iodide ion with hydrogen peroxide catalyzed by RHRP

Form of enzyme	Rate constant	
	$k_1/\mu M^{-1}\,s^{-1}$	$k_2/\mu M^{-1}\,s^{-1}$
RHRP	0.8	7.0
F143E	0.014	0.30

Note. Conditions: 0.1 *M* Na-acetate buffer, pH 5.0, 25 °C. Error does not exceed 10 % from the value determined.

sults in retardation of the stage of oxidation of ABTS (k_1 is 23 % and k_3 is 5.7 % in relation to the corresponding values for RHRP). The monomolecular rate-determining stage (k_4) for the F143E mutant becomes a governing factor, because the k_4 value falls by more than two orders of magnitude in comparison with that of RHRP.

The kinetic data on the oxidation of the iodide ion (Table 3) show that the mutation significantly affects both rate constants. A comparison of the substrate specificity of RP and tobacco NP reveals that the mutation affects the activity towards all of the substrates studied. The profile of substrate specificity towards ABTS remains generally unchanged, but the activities towards phenol and guaiacol decrease more strongly than the other activities, and the activity towards *o*-dianisidine increases by 3 times. It should be noted that *o*-dianisidine is the best substrate for tobacco peroxidase.[12]

The data obtained are in accordance with the assumption on localization of the phenylalanine residue. Replacing the nonpolar residue with a negatively charged glutamic acid residue should produce electrostatic hindrances at the entrance to the active center for negatively charged molecules, hemin itself and many other donor substrates among them. The decrease in the apparent rate constant of the monomolecular stage of elimination of the product of ABTS oxidation (k_4) by two orders of magnitude for the F143E mutant additionally attests to the interaction of the radical-cation of the oxidation product of ABTS with the glutamic acid residue at the entrance to the active center.

The data on radiation inactivation of the mutant (Fig. 3) demonstrate the appearance of an induction period comparable with that observed for the native enzyme due to the protecting effect of polysaccharide fragments in the enzyme.[13] Apparently, the mutation produces electrostatic hindrances to penetration of radiation-induced OH-radicals and hydrated electrons (e^-_{aq}) into the active center. The ratios of the radiation-chemical yields (G) of the active products of water radiolysis, which are significant for inactivation of HRP, are: $G(OH)$: $G(e^-_{aq})$: $G(H_2O_2)$ = 2.7: 2.7: 0.7, respectively. Replacement of the hydrophobic Phe residues by ionogenic His (RHRP-F41H[14]) and negatively charged Glu (RHRP-F143E) results in an increase in the radiation stability in both cases, especially at initial stages of

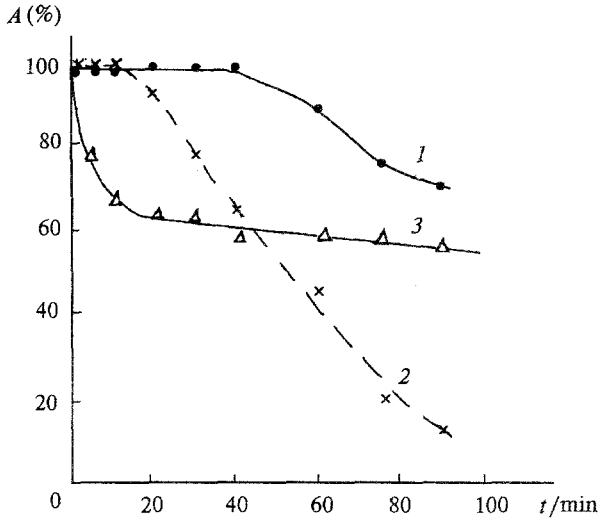

Fig. 3. Decrease in catalytic activity of RHRP-F143E towards the iodide ion (*1*), ABTS (*2*), and *o*-phenylenediamine (*3*) in relation to the irradiation dose.

inactivation, when conformational changes in the area of the active center are the main factor. The appearance of an induction period and an increase in D_{80} (the dose at which 80 % of the molecules retain enzymatic activity) were observed. Simultaneously, the stability of the F143E mutant increases by *ca.* 35 % because e^-_{aq} cannot penetrate into the active center.

It should be noted that the binding site of *o*-phenylenediamine is protected to a smaller extent than the binding sites of the iodide ion and ABTS. As can be seen from the model of the active center, Phe142, which is involved in binding aromatic substrates, is closer to the entrance of the active center than Phe143, and, hence, is protected to a lesser extent under replacement of F143E against the attack of radicals generated outside the molecule.

Generalizing the experimental data on the preparation, purification, kinetics, and radiation characteristics of the F143E mutant form of horseradish peroxidase, one can assume that the point mutation performed does not affect the substrate-binding site of the peroxidase, since it causes a decrease in catalytic activity towards all of the substrates, *i.e.*, it affects the structure of the active center as a whole, producing electrostatic hindrances at its entrance.

Experimental

Ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), *o*-phenylenediamine, isopropyl-β-D-thiogalactopyranoside (IPTG), sodium dodecylsulfate (SDS), trimethylaminomethane (Tris), oxidized glutathione, diiodothreitol (DTT), hemin (Sigma, USA), bactotriptone, and yeast extract (Difco) were used. Restrictases, ligases, and other enzymes for mutagenesis, including a Sculptor™ IVM System kit for

Table 4. Substrate specificity of the peroxidase

Substrate,	Peroxidase			
E/mg	NHRP	RHRP	F143E	Tobacco
ABTS	2000	4000	50	650
Guaiacol	43	20	7	53
Ferrocyanide	123	190	330	480
Phenol/antipyrine	59	20	7	2.4
o-Dianisidine	37	10	34	66
o-Phenylenediamine	73	28	16	18
KI	20	9	32	1.1

Note. The activity towards ABTS (*E*/ mg) is assumed to be 100 %.

directed mutagenesis and the original pSA261 plasmide with the HRP gene under control of a tac-promotor were kindly supplied by Amersham International plc (UK).

The measurements of the peroxidase activities were carried out on a Shimadzu UV 120-02 spectrophotometer (Japan) at 25 °C according the procedures described below.

For ABTS. An ABTS solution (0.05 mL, 15 mmol L^{-1}) and an aliquot of the enzyme were added to NaOAc buffer (2 mL, 0.1 *M*, pH 5.0); the reaction was initiated by the addition of 0.5 % H_2O_2 (0.1 mL). The extinction coefficient at 450 nm was taken as 36800 L mol^{-1} cm^{-1} (Ref. 15). The activities were represented in *E* units (μmol min^{-1}) per 1 mg of protein.

For KI. A KI solution (0.05 mL, 11.6 mmol L^{-1}) and an aliquot of the enzyme were added to NaOAc buffer (2 mL, 0.1 *M*, pH 5.0); the reaction was initiated by the addition of 0.5 % H_2O_2 (0.1 mL). The extinction coefficient (350 nm) was taken as 26000 L mol^{-1} cm^{-1}.

***o*-Phenylenediamine**. A solution of *o*-phenylenediamine (0.05 mL, 16 mg mL^{-1}) and an aliquot of the enzyme were added to 0.1 *M* Na-acetate buffer (pH 5.0) (2 mL). The reaction was initiated by the addition of a 0.5 % hydrogen peroxide solution (0.1 mL). The extinction coefficient at 445 nm was taken as 11100 L mol^{-1} cm^{-1}.

The apparent rate constants of the oxidation reactions of ABTS and the iodide ion for RHRP and NHRP were measured from the data on stationary kinetics, with concentrations of the substrates in the following ranges: 0.015—0.15 m*M* of ABTS, 0.5—5 m*M* of KI, 0.010—0.1 m*M* H_2O_2, and 0.5—80 m*M* of the enzymes. The H_2O_2 concentration was measured using absorbance at 240 nm, (ε = 43.6 L mol^{-1} cm^{-1})[2], and the ABTS and KI concentrations were determined by the weight of a batch.

The recombinant enzyme was prepared using the procedure described previously.[13]

Mutant F143E HRP was obtained by a method of directed mutagenesis of one-chain DNA. To replace the sequence TTC(Phe-142)-TTC(Phe-143)-ACT(Thr-144) by TTC(Phe-142)-GAA(Glu-143)-ACT(Thr-144) one 31-oligomer 5'-CTA-CCG-GCG-CCA-TTC-GAA-ACT-CTA-CCA-CAAC-3' with the restriction site Bsp 119-I (TTCGAA) supplying the primary selection of the mutant was synthesized. The primer was synthesized with an Applied Biosystems 380B synthesizer, and the final constructions were obtained with an Applied Biosystems 370A sequenator using Taq-polymerase and dye-labeled primers.

Synthesis, purification, and characterization of the HRP mutant forms were performed using procedures similar to those used for preparation of RHRP.

The heme content was measured using the formation of pyridine-hemochromogene.[17] Protein content was determined spectrophotometrically.[18] The homogeneity of the enzyme preparations was tested by electrophoresis in polyacrylamide gel in the presence of SDS.

Irradiation of the solution of HRP mutant was performed using a γ-source (P_γ 0.05 Gy s^{-1}), taking into account spontaneous and post-irradiation inactivation.

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