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Wild-Type and Mutant Forms of Recombinant Horseradish Peroxidase C Expressed in *Escherichia coli*

Substrate Specificity and Stability Under Irradiation

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ABSTRACT

Two horseradish peroxidase C (HRPC) mutants with substitutions in the active center, i.e., Phe41→His and Phe143→Glu, were compared to the wild-type recombinant enzyme expressed in *Escherichia coli* in terms of the enzymatic activity and stability under irradiation. Both mutations caused a significant decrease in activity, but it was still possible to follow the effect of mutations on the key steps of the reaction mechanism. Phe41 can be considered a nonpolar barrier separating histidine residues in the active center and providing a firm noncovalent binding with the highly hydrophobic porphyrin ring. The replacement of Phe41 with the ionizable His residue destabilizes the enzyme. The Phe143→Glu replacement creates a negative charge at the entrance of the heme-binding pocket, and protects the latter from both donor substrates and free radicals.

The radiolytic inactivation of the wild-type and mutant forms of recombinant HRP suggested different binding sites for iodide, 2,2′-bis(3-ethylbenzothiasoline-6-sulfonate (ABTS), guaiacol, and *o*-phenylene diamine. The study of kinetics and inactivation is in agreement with the direct binding of iodide to the heme porphyrin ring. The results also suggest that the ABTS binding site is less accessible than that for *o*-phenylene diamine.

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Index Entries: Site-specific mutagenesis; rate constants; hydrogen peroxide; ABTS; iodide; substrate specificity; substrate binding sites; inactivation; low-dosage radiolysis.

INTRODUCTION

Heme-containing peroxidases are widely spread in nature and of great importance from both practical and theoretical points of view. The crystal structure data for heme-containing plant and fungal peroxidases (cytochrome C peroxidase [*1*], manganese [2] and lignin peroxidases [3], and fungal peroxidase from *Coprinus cinereus* [*4*]) together with the alignments of the amino acid sequences (*5*) allow the construction of a model of the active center of horseradish peroxidase C (HRP C) (*6*) (Fig. 1). Plant peroxidases (EC 1.11.1.7) and HRP C, in particular, are known their wide substrate specificity, which could be ascribed to the specific binding sites for different donor substrates.

The investigation of molecular determinants of the substrate specificity of HRP C became possible because of its gene cloning and expression in *Escherichia coli* (*7,8*). The present study describes the enzymatic activity and stability under irradiation of two HRP C mutants with the substitutions in the active center, i.e., Phe41 → His and Phe143 → Glu. Phe41 is located between His40 and His42. The latter is coordinated to the heme iron (Fig. 1). Phe143 is located near the entrance to the heme-binding pocket in the vicinity of the binding centers proposed for guaiacol and iodide. Its replacement by a negatively charged glutamate could create the electrostatic hindrance for the electron donors and prevent their penetrating inside the active center. The results reported here confirm the hypothesis on the distinct binding sites for various substrates and emphasize the role of Phe41 and Phe143 in the catalytic mechanism.

MATERIALS AND METHODS

Recombinant HRP and its two single-point mutants, F41H and F143E, have been reactivated from the *E. coli* inclusion bodies by the refolding procedure developed in our laboratory (*9*) yielding 25 mg of the homogeneous active enzyme/1L of *E. coli* culture. The homogeneity was tested by SDS-PAGE.

Irradiation was performed as follows. Aliquots of peroxidase aqueous solutions were placed into test tubes and irradiated for certain time intervals by the γ-source with the power of P_γ = 0.05 gy/s. The irradiation dose (D) is expressed in gy throughout. The peroxidase sample from each tube was used for spectral studies and measuring the activity toward different substrates. All radiolysis experiments were performed in triplicate at 22°C in 0.01*M* Tris-HCl buffer, pH 8.0.

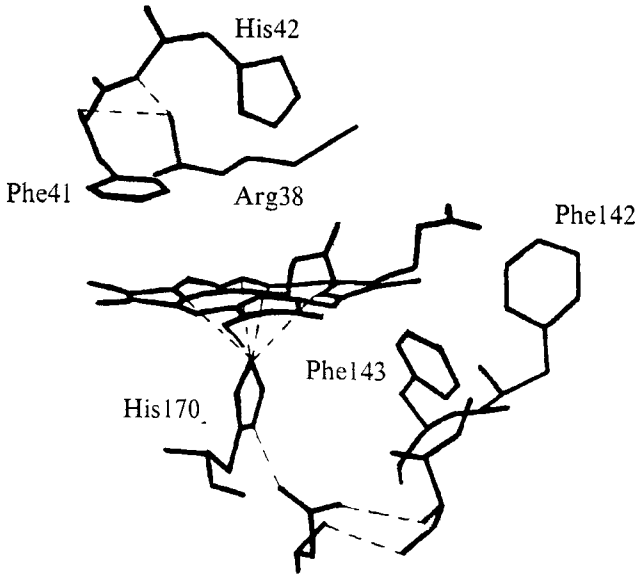

Fig. 1. Model of the active center of HRP C.

Measurements of peroxidase activity were performed on a Shimadzu UV 120-02 spectrophotometer using diammonium salt of 2,2′-azino-bis(3-ethylbenzothiasoline-6-sulfonate (ABTS) (*10*), phenol/4-aminoantipyrine (P-A) (*11*), guaiacol (Gua) (*12*), *o*-dianisidine (*o*-DA) (*13*), *o*-phenylene diamine (*o*-PD) (*14*), potassium ferrocyanide $[Fe(CN)_6]^{4-}$ (*15*), and potassium iodide (KJ) (*16*). The activity is given as the standard enzyme units (μ mol/min/mg protein) and converted into the turnover number (s^{-1}) by dividing the former by the heme content determined by the pyridine-hemochromogen method (*17*).

The apparent rate constants for the ABTS oxidation catalyzed by native and recombinant HRP C were determined from the steady-state kinetic measurements in the concentration range 0.005–0.1 m*M* for H_2O_2 and 0.004–0.04 m*M* for ABTS. The apparent rate constants for the iodide oxidation were calculated in the same way at H_2O_2 and iodide concentrations 0.01–0.1 and 0.5–5.0 m*M*, respectively. The concentration of H_2O_2 was determined at 240 nm (molar absorptivity $43.6M^{-1}$/cm).

RESULTS AND DISCUSSION

The study of structure–function relationships for HRP C using the *E. coli* expression system is complicated by artificial folding of the nonglycosylated HRP C polypeptide. The recombinant enzyme is less stable both under elevated temperatures and in the course of enzymatic reaction (*8*). The investigation of the enzyme under radiolysis (Fig. 2) indicates also its low stability compared to the native HRP C. The radiolytic inactivation of

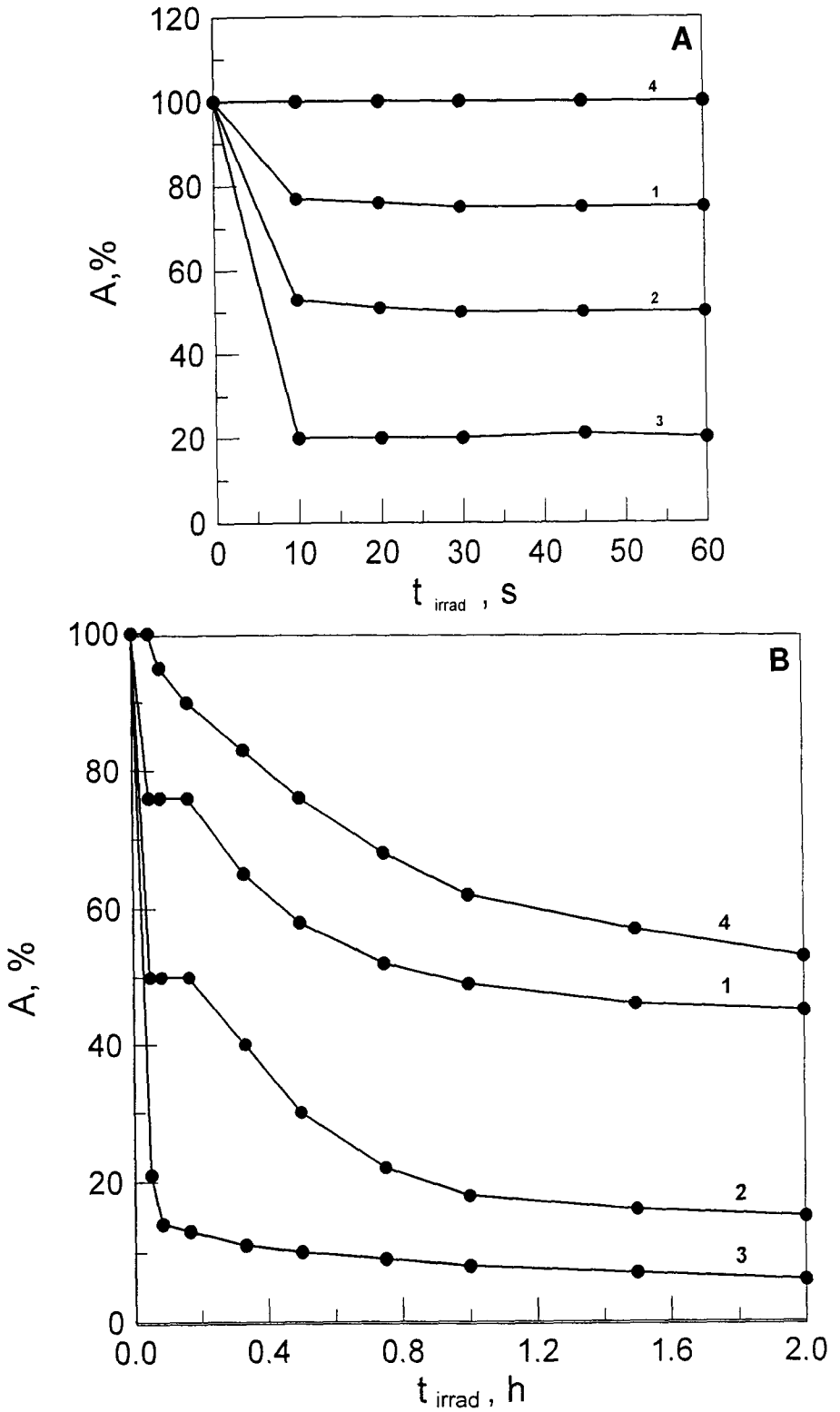

Fig. 2. Changes in the catalytic activity of wild-type recombinant HRP C induced by the irradiation doses less (**A**) and more (**B**) than 3 gy. Concentration of recombinant HRP C, μM: 1–1, 2–0.1, 3–0.01. Inactivation curve (4) for native HRP C (0.1 μM) is presented as a reference.

Table 1
Substrate Specificity of the Recombinant Wild-Type, F41H and F143E Mutant HRP C Forms Produced in *E. coli*

	HRP_{rec}	F41H	F143E
Heme content, %	98	80	45
Specific activity U/mg, toward			
ABTS	4000 (100%)	90 (2.2%)	50 (1.2%)
P-A	230 (100%)	3 (1.3%)	3 (1.3%)
Gua	260 (100%)	0	3 (1.3%)
o-DA	370 (100%)	7 (1.9%)	65 (17.0%)
$[Fe(CN)_6]^{4-}$	7530 (100%)	270 (3.5%)	165 (2.2%)
KJ	870 (100%)	29 (3.3%)	17 (1.8%)
o-PD	940 (100%)	19 (1.9%)	9 (0.9%)

the native HRP C is characterized by a lag period at the initial step of inactivation. The lag period for the native HRP C ascribed to the protective role of oligosaccharide chains (*18*) was not observed for the recombinant HRP. The substrate specificity and kinetic properties of the latter were also altered (*19*).

We have recently shown (*9*) that the refolding itself and, in particular, the entrapment of heme are affected by amino acid replacements. The entrapment into the F41H and F143E HRP mutants occurs fast (5 h) and slow (80 h), respectively (*9*), indicating a role of single-point mutations on the protein folding. Thus, if the *E. coli* expression system is used to produce the HRP C recombinant forms, one should take into account that a mutation will affect both the folding and the mechanism.

Catalytic properties of the HRP C mutants obtained were dramatically changed (Table 1). There was a significant loss in catalytic activity. It means that both mutations affect the whole structure of the active center. The only difference to be emphasized is that the F143E mutant preserves about 17% activity toward *o*-DA, whereas all other activities drop to 2–3% level.

To distinguish the mutation effects on the hydrogen peroxide heterolytic cleavage and the ABTS oxidation, the apparent rate constants k_1 and k_2 have been determined from the steady-state kinetic data (Table 2). The rate constants were compared to those obtained previously by Smith et al. (*20*) for the HRP C_{rec} and F41V mutant. The catalytic cycle in the case of single-electron donors (ABTS) is represented by the scheme:

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

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

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

$$E\text{-}P \rightarrow E + P\ (k_u)$$

where E, EI, and EII are native ferric enzyme, Compound I and Compound II, respectively, and E-P is an enzyme–product complex.

Table 2
The Apparent Rate Constants for ABTS Oxidation by the Recombinant Wild-Type and Mutant HRP C (0.1*M* Na-acetate Buffer, pH 5.0, 25°C)

HRP_{rec}	Specific activity, U/mg	Rate constants		
		k_1, $\mu M^{-1}/s$	k_3, $\mu M^{-1}/s$	k_u, $\mu M^{-1}/s/s$
HRP wild-type	4000	4.8	3.0	4600
F41H	90	0.022	0.37	—
F143E	50	1.1	0.32	35
HRP wild-type (*20*)	880	5.9[a]	3.7	850
F41V (*20*)	180	0.63	0.81	90

Errors are < 10%.
[a]The Error is 50%.

Table 3
The Apparent Rate Constants for Iodide Oxidation by Recombinant Wild-Type and Mutant HRPs (0.1*M* Na-acetate Buffer, pH 5.0, 25°C)

HRP_{rec}	Rate constants		
	k_1, $\mu M^{-1}/s$	k_2, $\mu M^{-1}/s$	k^a, M^{-2}/s
HRP wild-type	0.8	7.0	—
F143E	0.014	0.3	—
F41H[a]	—	—	2.5×10^5

Errors are < 10%.
[a]Third-order rate constant.

The rate constants for the wild-type HRP C forms obtained here (4000 U/mg) and elsewhere (850 U/mg) (*20*) show that the preparations have similar kinetic characteristics. The difference in the specific activity toward ABTS can be ascribed to a fivefold increase of the rat-elimiting k_u.

The F41H mutation affects mainly the k_1, decreasing it by a factor of 100. In the case of F41V (*20*), the replacement affected k_1 also. The unimolecular rate-limiting dissociation (k_u), which was determined according to ref. (*20*), is no more crucial for the F41H mutant. This contrasts with other recombinant HRP C forms, such as F143E in particular. A decrease of k_u by two orders of magnitude for the latter in the case of ABTS gives additional evidence for the enhancement of the ABTS cation-radical product interaction with the enzyme in the presence of the negatively charged glutamate residue. The F143E substitution affects also the values of k_1 and k_3 for ABTS, which are 23 and 5.7% of those for the wild-type recombinant enzyme, respectively. We can also speculate that the F143E substitution decreases the accessibility of the ABTS binding site.

The kinetic study of iodide oxidation (Table 3), which is the only two-electron donor among the substrates tested (*see* the scheme *below*), shows the significant changes in the reaction mechanism for the F41H mutant.

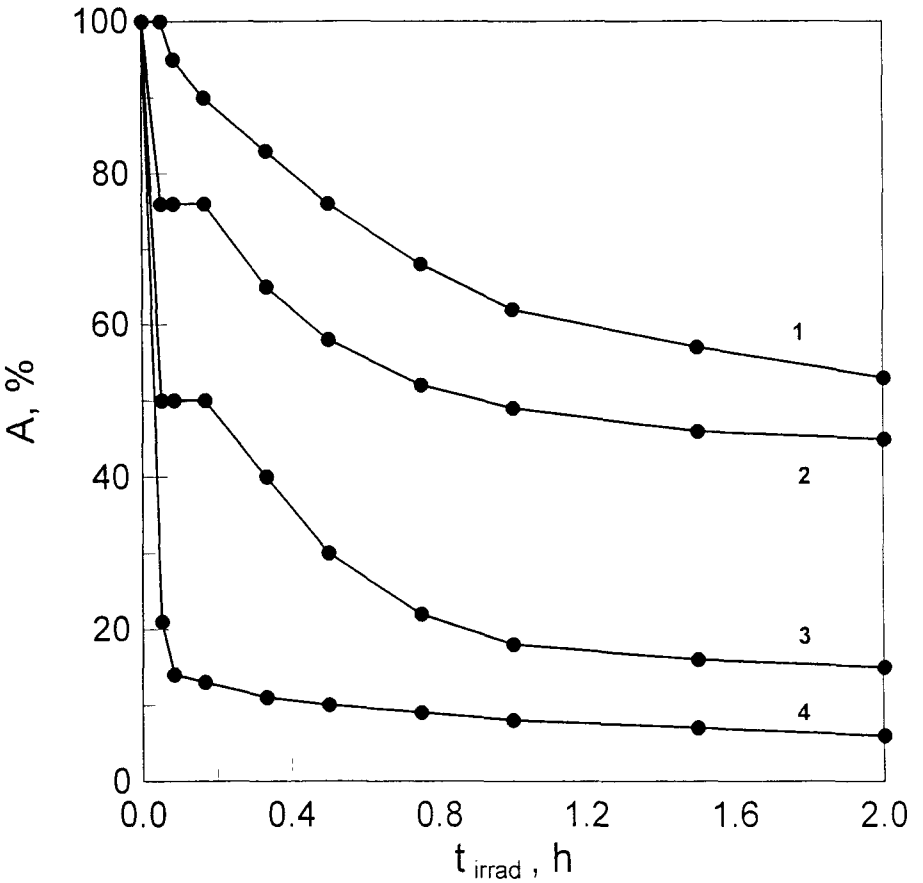

Fig. 3. Changes in activity of the wild-type recombinant HRP C with respect to iodide (1), phenol (2), guaiacol (3), and ABTS (4) induced by irradiation.

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

$$EI + J^- + H^+ \rightarrow E + HOJ \ (k_2)$$

The oxidation catalyzed by F41H does not follow the Michaelis-Menten kinetics and the rate law is $v = k^*$ [E][H_2O_2][KJ] where k^* is the third-order rate constant. This formally corresponds to the ternary interaction and allows a direct interaction of iodide with the heme porphyrin ring to be proposed.

The F143E replacement affects both rate constants, but has no effect on the reaction mechanism. The reaction mechanism follows Michaelis-Menten kinetics (parallel lines in double reciprocal plots) for the recombinant wild-type HRP C and F143E mutant.

The radiolytic inactivation applied to native HRP C (*18*) demonstrated the presence of distinct binding sites for guaiacol, phenol, ABTS, and iodide. This is in accordance with the conclusions of Hartmann and Ortiz de Montellano deduced from the studies of heme modification (*21*). The effects of irradiation on enzymatic activity of the recombinant enzyme with respect to various electron donors (Fig. 3) confirmed the above con-

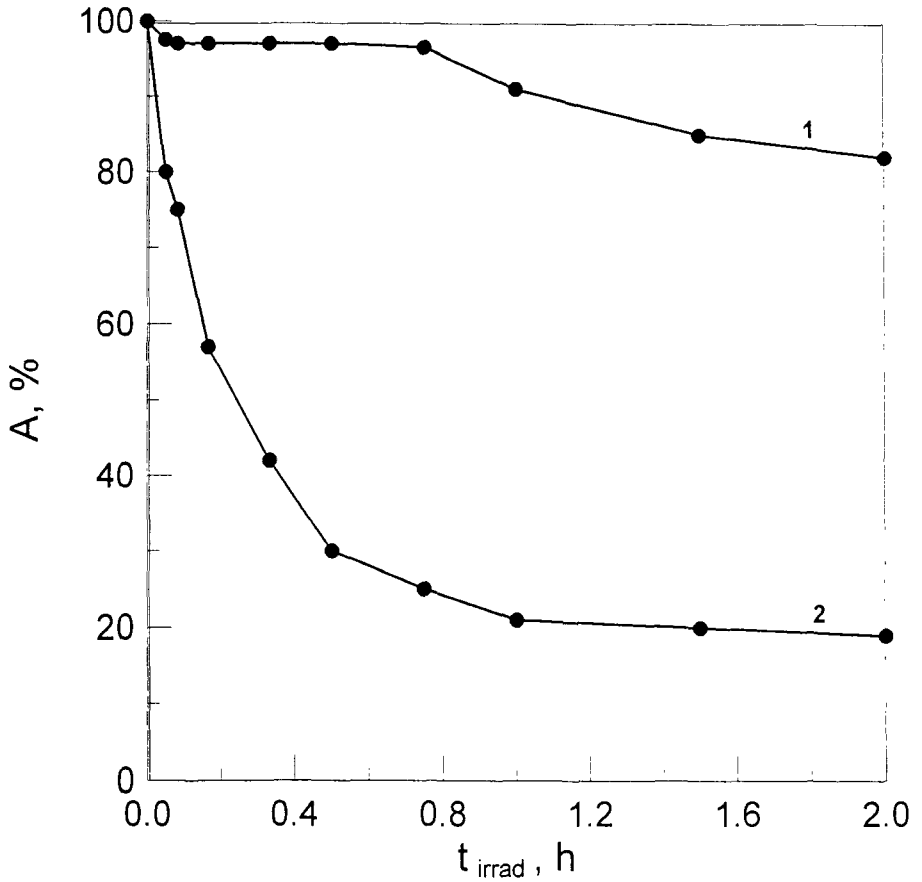

Fig. 4. Changes in activity of the F41H mutant of the recombinant HRP C with respect to iodide (1) and ABTS (2) under irradiation conditions.

clusion. The activity toward iodide was the most persistent in contrast to that toward ABTS and guaiacol. One can interpret it as a direct interaction of iodide with the heme porphyrin ring, and such a proposal is in agreement with the above ones based on kinetic studies.

Inactivation curves for the recombinant enzyme (Fig. 4) indicate also the existence of a dynamically stable conformation. This fact could be related to the existence of a "molten globule" state widely used in folding/unfolding and inactivation studies (*22*). It refers to the folding intermediate with a stable secondary structure. The substrate specificity of this conformation differs significantly from that of the wild-type recombinant HRP. The ratio of the activities toward iodide and phenol to that of ABTS for this dynamically stable "molten globule" is 2 and 1.6 times higher compared to the initial ones for the original preparation of the recombinant HRP C (calculated from the data presented in Fig. 4). The substrate specificity of the recombinant HRP C "molten globule" corresponds to that of the native enzyme (*19*). Taking into account that native HRP C was isolated from the living plant cells and thus, was partially inactivated,

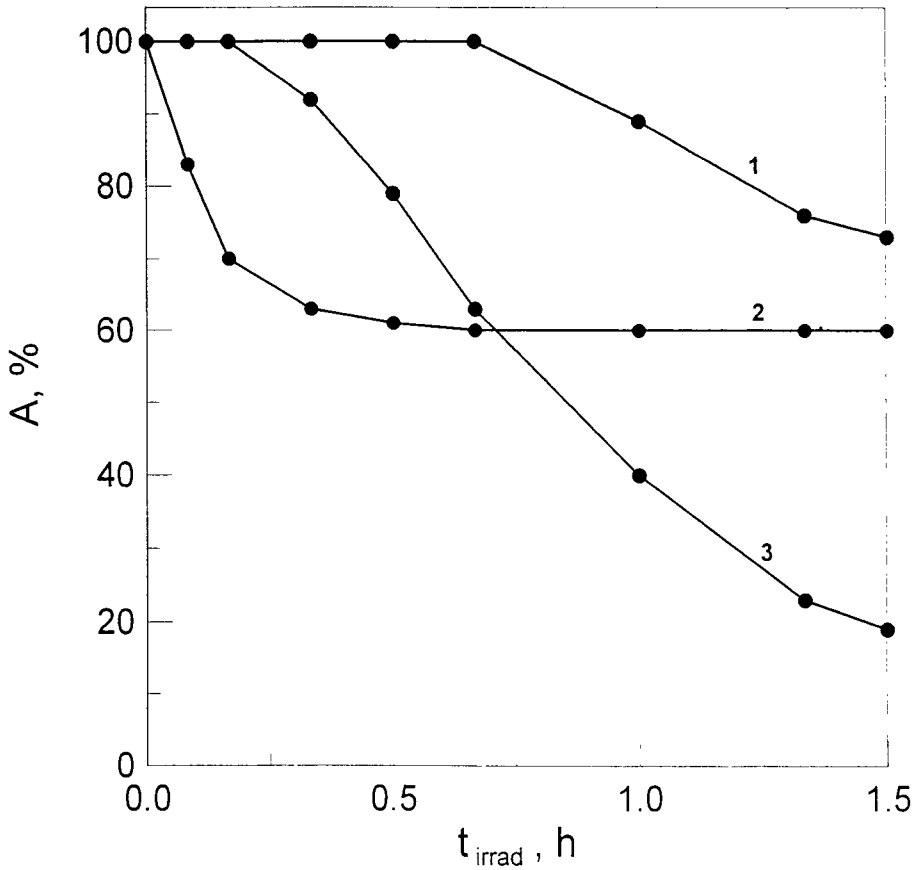

Fig. 5. Changes in activity of the F143E mutant of recombinant HRP C with respect to iodide (1), *o*-PD (2), and ABTS (3) under irradiation conditions.

it could be proposed that mechanisms of inactivation under irradiation and in the reaction course might be similar.

The data on radiolytic inactivation of the F41H mutant (Fig. 4) also gives evidence for the binding of iodide to the porphyrin ring because only this activity is resistant to irradiation. The most interesting results associated with a partial stabilization of the F143E mutant against irradiation are shown in Fig. 5. The negatively charged glutamic acid at the entrance to the heme-binding pocket leads to the stabilization of the iodide and ABTS activities against the irradiation. We can speculate that the ABTS binding site is protected by the Phe143 residue in contrast to the *o*-PD binding site, which is still subjected to the radical attack in the F143E mutant.

CONCLUSIONS

The results obtained confirm the essential role in catalysis of the replaced residues Phe41 and Phe143. The presence of charged His41 in the

F41H mutant instead of hydrophobic Phe, Val, or Trp in mutants described earlier (*20*) results in a significant drop in the catalytic activity and a change in the rate-limiting step. Phe41 can be considered as a nonpolar barrier that separates the histidine residues in the active center and provides a firm noncovalent binding with the hydrophobic porphyrin ring. The radiolytic inactivation of the wild-type and mutant forms of the recombinant HRP C demonstrated different binding sites for iodide, ABTS, guaiacol, and *o*-PD. The data on kinetics and radiolytic inactivation are in agreement with the proposed direct binding on iodide to the heme porphyrin ring. The results also allow the assumption that the ABTS binding site is less accessible than that of *o*-PD. Thus, the HRP C site-specific mutagenesis coupled with kinetic and inactivation studies gives a structural information on substrate binding sites and can be used as a tool to study effects of the protein environment on catalytic properties of heme.

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