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The role of tryptophan residues in radiation sensitivity of enzymes as exemplified in horseradish peroxidase

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The stability of recombinant wild-type horseradish peroxidase and its tryptophan-less mutant Trp117Phe toward γ-radiation was studied. The absence of tryptophan in the enzyme molecule results in a certain stabilization, which is manifested as the absence of the initial drop in activity and appearance of a lag period for doses of up to 45 Gy. Contrary to the wild-type enzyme, the dose response of the mutant is almost independent of the nature of the substrate used to measure the catalytic activity; this indirectly indicates that Trp117 participates in the oxidation of substrates. Pretreatment of the wild-type recombinant enzyme with hydrogen peroxide destabilizes the enzyme towards irradiation, while the same procedure for the mutant enzyme has virtually no effect on the dose response curve. This suggests the modification of Trp117 in the oxidation of the native enzyme with H_2O_2 in the absence of electron-donor substrates, which is the modification of Trp171 in the recombinant lignin peroxidase.

Key words: radiation-induced inactivation, recombinant horseradish peroxidase, mutagenesis, Trp117Phe, modification, hydrogen peroxide.

The radiation-induced inactivation of enzymes on irradiation of their aqueous solutions is due to the damage effect of the water radiolysis products, mainly the $H^{\cdot}$ and $^{\cdot}OH$ radicals and solvated electrons.[1] It is known[2,3] that at low radiation doses, the reactions of these species with the Trp, Tyr, and His residues are the most significant. This results in conformational changes, which determine the subsequent radiation stability of enzymes and other physicochemical properties.

Heme-containing plant peroxidases incorporate only one highly conservative Trp residue.[4] This residue (Trp117 for the horseradish peroxidase) occurs in the distal area of the enzyme at a distance of 8—9 Å from the heme (Fig. 1) and can perform both structural and catalytic functions.[5]

The purpose of the present work was to construct a tryptophan-less mutant (Trp117Phe) horseradish peroxidase (HRP) and to compare its radiation sensitivity with that of the wild-type recombinant horseradish peroxidase (RHRP).

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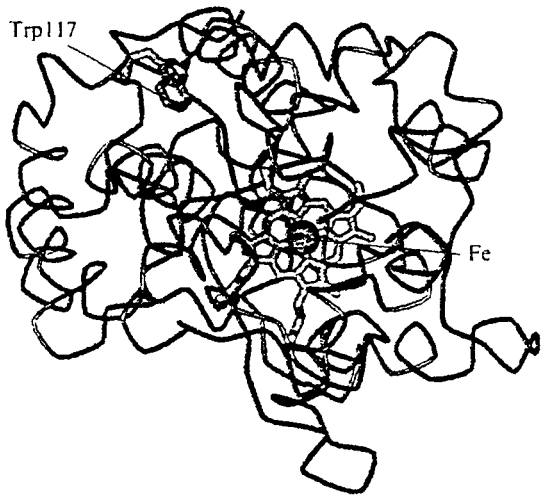

Fig. 1. Crystal structure of the recombinant wild-type horseradish peroxidase from the distal region. The conservative Trp117 residue is shown.

Results and Discussion

In our previous communications,[6,7] we showed that the radiation sensitivity and substrate specificity of RHRP differ from those of the native HRP (NHRP). The dose response of the RHRP includes an initial decrease in the enzymatic activity and a plateau for doses of 10—45 Gy, which implies the occurrence of a stable intermediate. In the case of the mutant form Trp117Phe, the initial decrease in the activity is not observed and a distinct lag-period appears (Fig. 2). This observation is valid for all the substrates used and reflects the structure differences between the mutant and the wild-type enzymes, namely, the absence of a stable intermediate for the former. It should be noted that the molecular weight (34 kDa) and biochemical characteristics of the mutant (*RZ** (3.0) and the specific activity in the peroxidase reaction (Table 1)) are virtually the same as those for RHRP. Thus, this difference cannot be attributed to the changes in folding of the mutant. Thus, the single replacement (Trp117Phe) is the reason, first, for the noticeable stabilization of the enzyme towards indirect action of irradiation and, second, for its inability to form the stable intermediate, typical of RHRP.[8]

The substrate specificity of the mutant has also changed; the dose response curves for ammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), guaiacol, and *o*-dianisidine, used as substrates, virtually coincide (Fig. 2). This result differs fundamentally from that obtained for RHRP,[7,8] for which ABTS was found to differ substantially from the other substrates and whose activity towards ABTS proved to be more sensitive to irradiation.[7,8] The fact that the shape of the corresponding curves for the tryptophan-less mutant does not depend on the nature of the substrate attests indirectly that the Trp residue is involved in the peroxidase-catalyzed oxidation of ABTS (or that this residue influences functioning of the ABTS-binding site).

A specific feature of the RHRP is that the enzyme under study did not participate previously in any catalytic cycles, whereas in the case of native RHRP, modification of some amino acid residues during the enzymatic reaction cannot be ruled out.

In the absence of an electron donor in the oxidation with hydrogen peroxide, the enzyme molecule itself can act as the reducing agent. In other words, the so-called compound I, containing iron and a porphyrin π-radical cation, which forms under the action of H_2O_2, is able to be reduced at the expence of oxidation of amino acid residues in the enzyme itself. Thus hydroxylation of Trp171 located at a distance of 16 Å from hemin on treatment with 3 equiv. of H_2O_2 was noted for recombinant lignin peroxidase.[9]

$$E\text{—}RH + H_2O_2 \rightarrow EI\text{—}RH + H_2O, \quad (1)$$

$$EI\text{—}RH \rightarrow EII\text{—}R^{\cdot} + H^{+}, \quad (2)$$

$$EII\text{—}R^{\cdot} + OH^{-} \rightarrow E\text{—}ROH, \quad (3)$$

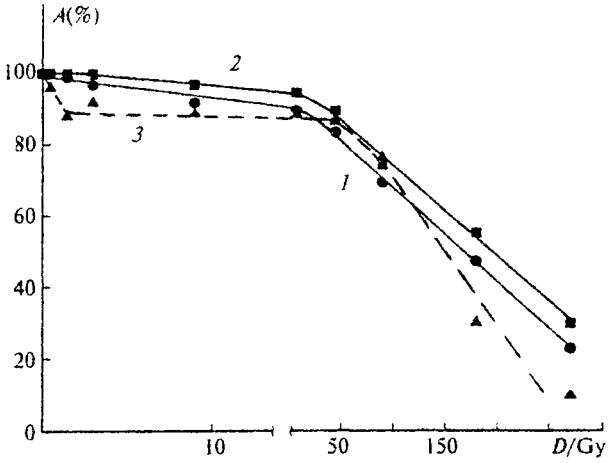

Fig. 2. Dose (*D*) response of the enzymatic activity (*A*) of the mutant RHRP Trp117Phe with the use of ABTS (*1*), guaiacol (*2*), and *o*-dianisidine (*3*) as substrates.

Table 1. Specific activity (*E*) of horseradish peroxidase preparations

Substrate	*E*/act.u mg^{-1}	
	RHRP	Trp117Phe mutant
ABTS	1500	1100
Guaiacol	550	700
o-Dianisidine	800	700

* The ratio of the optical densities at 403 and 280 nm in the electronic spectrum characterizes the saturation of the preparation with hemin, *i.e.*, its purity.

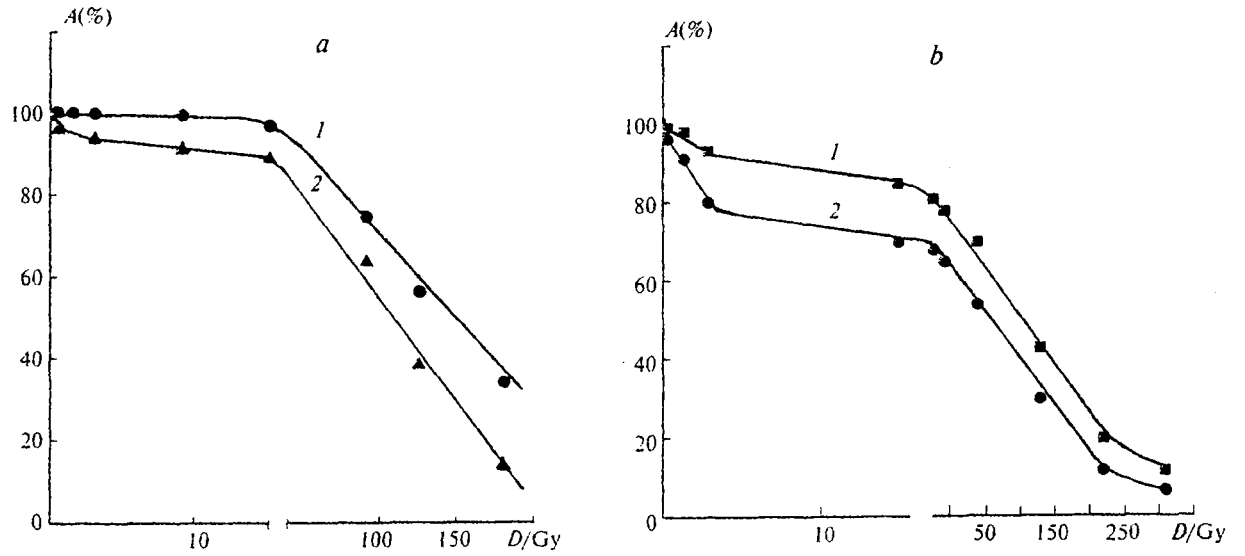

Fig. 3. Effect of pretreatment with H_2O_2 on the dose response of the mutant form Trp117Phe (*a*) and RHRP (*b*) for an irreversible enzyme (*1*) and an enzyme treated with an equimolar amount of H_2O_2 (*2*). The substrate is guaiacol.

where R˙ is the HRP radical, E—RH is the native enzyme containing no modifications, EI—RH is compound I derived from the nonmodified enzyme, EII—R˙ is compound II with the radical as a part of the protein, and E—ROH is the hydroxylated enzyme.

For one of the RHRP mutants, namely, His42Leu, the formation of the radical in the protein rather than in porphyrin was detected in the formation of compound I. It was suggested that Trp117 participates in the reduction of the active site.*

In view of these findings, we studied further the effect of treatment of the enzyme with an equimolar amount of hydrogen peroxide on the dose response of the enzymatic activity. It was found that the fundamental difference between the tryptophan-less mutant form and RHRP is that in the former case, pretreatment with H_2O_2 has virtually no effect on the radiation sensitivity of the mutant (Fig. 3, *a*), and in the latter case, it causes some destabilization of the enzyme (Fig. 3, *b*).

We have not obtained direct evidence for the modification of Trp117 on treatment with hydrogen peroxide for wild-type RHRP. However, the difference observed between the dose response of the enzymatic activity for this form and for the mutant form Trp117Phe suggests indirectly that the Trp117 residue participates in a process similar to that occurring in lignin peroxidase and represented by Eqs. (1)—(3). This conclusion is consistent with the fluorescence of the Trp residue of the enzyme during the catalytic oxidation of tyrosine with H_2O_2 detected in an early paper.[10] Further studies of the tryptophan-less mutant form of the enzyme would provide answers to numerous questions concerning the details of the mechanism of peroxidase-catalyzed oxidation. The results obtained here confirm convincingly the assumption made previously[11] that the role of Trp residues in the radiation effects on enzymes is so significant that, if this residue is the only one in the molecule, its role in the structure should be fundamental (related, apparently, to the flexibility of the active site).

* R. N. F. Thorneley, unpublished results.

Experimental

The following chemicals were used: ammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), guaiacol, *o*-dianisidine, isopropyl 1-thio-β-D-galactopyranoside, sodium dodecyl sulfate, tris(hydroxymethyl)aminomethane (Tris), oxidized glutathione, dithiothreitol, hemin (Sigma, USA), bactotryptone, and yeast extract (Difco). Restrictases, ligases, and other enzymes for mutagenesis and the initial pSA261 plasmid with the HRP gene under control of the *tac*-promoter were provided by the Amersham International plc company (UK).

The recombinant horseradish peroxidase was obtained by expression of *E. coli* by the refolding and purification procedures that we had developed previously.[12] The Trp117Phe mutant form of the enzyme was constructed similarly to the Phe41His and Phe143Glu mutant forms of horseradish peroxidases described previously using the oligonucleotide primer 5′-CCTAATGGTACTCGGAATGAGGGCCCGCCG-3′, which ensures replacement of Trp117 by Phe. The genetic construction was sequenced on an Applied Biosystems sequencer. Refolding of the mutant form from the *E. coli* inclusion bodies was performed by a procedure similar to that used for the wild-type recombinant enzyme with the following modifications. During refolding, ammonium sulfate (20% of the amount necessary for saturation) and hemin were added; the pH of the medium for the refolding was decreased from 9.3 to 8.5.

The catalytic activity towards a donor substrate was measured as described previously[13—15] using ABTS,[13] guaiacol,[14] and *o*-dianisidine.[15] Pretreatment with hydrogen peroxide was performed by incubation of the enzyme with an equimolar amount of H_2O_2 for 1 h.

Irradiation was carried out on a γ-radiation source with the dose rate $P_\gamma = 0.05$ Gy s^{-1} using 10^{-7} *M* solutions of enzymes in a 50 m*M* Tris—HCl buffer, pH 7.0. After irradiation, the solutions were kept for 1 h at 20 °C and the residual enzymatic activity was measured.

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