Comparative study of the inactivation of horseradish peroxidase under the effect of H_2O_2 and ionizing radiation

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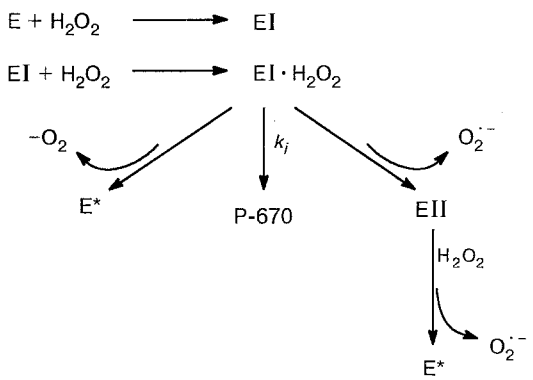
The inactivation of native and recombinant horseradish peroxidase in the presence of hydrogen peroxide and under ionizing radiation was studied. The types of peroxidase activity differ in sensitivity towards the inactivating effect of H_2O_2: the activity in relation to the iodide ion is more stable than the activity in relation to ammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and *o*-phenylenediamine. Similar inactivation was observed in the course of the radiolysis of peroxidase. It was assumed that the initial period of peroxidase inactivation in the presence of hydrogen peroxide has a radical nature and is related to the generation of superoxide radicals, which modify the protein moiety, resulting in the destruction of heme. The R-670 compound was not formed under the conditions studied. However, the E → EI transition occurred, depending on the radiation dose and the enzyme concentration.

Key words: recombinant horseradish peroxidase, native horseradish peroxidase, radiation inactivation, chemical inactivation.

Horseradish peroxidase (HRP) is one of the enzymes widely used in analysis, and hence the problem of its stability (in particular, the mechanism of its inactivation in the course of a reaction) has attracted the attention of researchers. Previously,[1,2] it has been shown that the main inactivating agent of HRP is the substrate-oxidant, *i.e.*, H_2O_2, and a hypothetical scheme of the inactivation has been proposed:



In accordance with this scheme three routes of inactivation can be realized: catalase, destructive (the disappearance of the Soret band and the appearance of the P-670 compound), and radical (the generation of superoxide-radicals). The data obtained to date do not allow one to estimate the contribution of each of the above routes because the influence of H_2O_2 on the activity of the enzyme was studied, as a rule, only towards one substrate. Also, the native HRP was usually used, which already "worked" in the living cell and, therefore, was modified in a specific way.

Advances in HRP gene expression in *E. coli* (see Refs. 3 and 4) allowed us to reconstruct the enzyme molecule from the intact protein part and hemin and thus to carry out experiments on "primary" inactivation.

Our previous studies on radiation inactivation of native and recombinant horseradish peroxidase (NHRP and RHRP, respectively)[5] and its Phe41 → His (F41H) mutant[6] demonstrated the difference between native and recombinant enzymes and the existence of different binding sites on the substrates. We concluded that the iodide ion interacted directly with heme, unlike ABTS. The oxidation of the latter needs an electron transfer chain. Since the mechanism of the radiation inactivation of the peroxidase is of a radical type due to the effect of the active products of the radiolysis of water (mainly, the OH radicals and the hydrated electrons, e^-_{aq}) it seemed important to compare the influence of H_2O_2 on the enzymatic activity of NHRP and RHRP measured using different substrates with the data on the radiation inactivation of these solutions.

Results and Discussion

The inactivations of NHRP and RHRP through the action of hydrogen peroxide are similar in character

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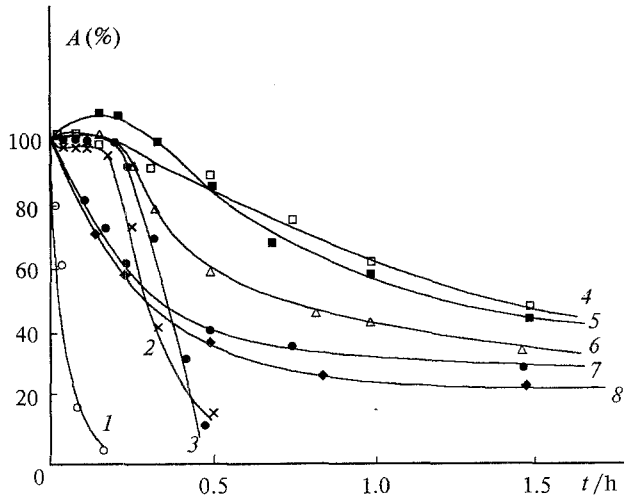

Fig. 1. Kinetics of the change in the enzymatic activity (A) of NHRP (10^{-7} mol L^{-1}) in 0.01 M Tris—HCl buffer (pH 8.0) during inactivation with H_2O_2 (5 mmol L^{-1}) (curves *1—3*) and ionizing irradiation (curves *4—8*); dose power P_γ = 0.05 Gy s^{-1}. Activities were measured towards KI (*1*, *4*), ABTS (*2*, *6*), *o*-phenylenediamine (*3*, *8*), phenol-antipyrine (*7*), and guaiacol (*5*).

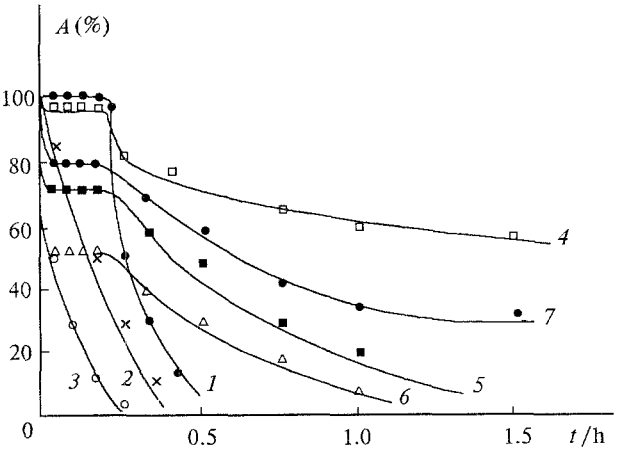

Fig. 2. Kinetics of the change in the enzymatic activity of RHRP (10^{-7} mol L^{-1}) in 0.01 M Tris—HCl buffer (pH 8.0) during inactivation with H_2O_2 (5 mmol L^{-1}) (curves *1—3*) and ionizing irradiation (curves *4—7*); P_γ = 0.05 Gy s^{-1}. Activities were measured towards KI (*1*, *4*), ABTS (*2*, *6*), guaiacol (*5*), phenol-antipyrine (*7*), and *o*-phenylenediamine (*3*).

(Figs. 1 and 2). Both enzymes practically retain their initial activity towards the iodide ion for 10 min (*i.e.*, an induction period exists), which can evidently be related to the fact that hemin remains intact, since, as has been discussed previously,[5] this substrate reacts directly with the porphyrin ring. After the induction period is finished, fast and irreversible inactivation proceeds. The activities of NHRP and RHRP determined in relation to ABTS differ in some extent. In this case, the recombinant enzyme appears more sensitive to H_2O_2 than the native

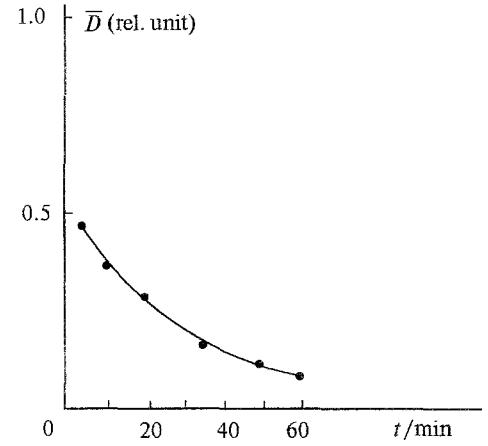

Fig. 3. Change in the content of the EI compound determined by absorbance ($\bar{D}$) at λ = 419 nm from the duration of the inactivation of NHRP ($5 \cdot 10^{-6}$ mol L^{-1}) in 0.01 M Tris—HCl buffer (pH 8.0) with H_2O_2 (50 mmol L^{-1}) ($\bar{D}_{NHRP}$ = 0.52, λ = 402 nm).

HRP (see Figs. 1 and 2). In addition, during inactivation of NHRP a small induction period was observed. It was smaller than that in the determination of the activity in relation to KI. The existence of the induction period is probably associated with two factors: the presence of carbohydrate fragments in NHRP[7] and the appearance of inactive molecules due to modification of the native enzyme *in vivo*.[5,8] The latter may cause a slight increase in the stability of NHRP (in comparison with RHRP) represented as a percentage in relation to the initial activity, because the native enzyme is less active than the recombinant enzyme in the absolute value. Finally, it should be noted that the binding site for *o*-phenylenediamine is the most sensitive to H_2O_2, especially in the case of RHRP.

The spectral studies show that a very fast transition of the enzyme into the EI compound (oxyferryl-enzyme) with λ_{max} = 419 nm takes place (for NHRP λ_{max} = 402 nm). The EI compound is irreversibly destroyed without reduction of the initial form, E, in 1 h (Fig. 3). The appearance of the so-called P-670 compound during the inactivation of NHRP as a result of cleavage of the bond between hemin and the protein and the destruction of heme has been described previously.[2] However, under our conditions its formation was not observed. One cannot exclude the possibility that the appearance of P-670 depends on the concentration of H_2O_2 and the concentration of the active form of the enzyme.

Based on the published data on heme-containing proteins[10] and the data from our investigations, the mechanism of the radiation-induced inactivation of HRP involves the modification of the protein globule, primarily of aromatic amino acid residues and histidine, by OH radicals, and a simultaneous attack by hydrated electrons on hemin. In the case of e^-_{aq}, changes in the

heme itself and, hence, changes in the activity of HRP in relation to KI should be observed. The OH radicals more strongly affects the conformation and the coordination of hemin with the protein moiety, and, at first, their action must result in a change in the activity in relation to ABTS. Since significant radiation sensitivity in the case of ABTS as the substrate and a prolonged induction period (the range of stability) in relation to KI (see Figs. 1 and 2) were observed, one can assume that the contribution of e^-_{aq} to the inactivation of both NHRP and RHRP cannot be a governing factor.

Considering the differences between the radiation inactivations of NHRP and RHRP, it should be stressed that RHRP has another conformation resulting from folding of its molecule[8] that only slightly affects the activity of the enzyme in relation to KI, but has a significant influence on its activity in relation to ABTS. In fact, in the case of RHRP the induction period is absent, and at doses <10 Gy 50 % of its activity is lost.

The data of the spectral studies indicate the formation of the EI compound both during radiation inactivation and during inactivation with H_2O_2. Note that when the concentration of enzyme in the solution is 10^{-6} mol L^{-1} it totally transforms to EI, and when the concentration of HRP is increased to $2.5 \cdot 10^{-5}$ mol L^{-1} the transformation E $\rightarrow$ EI is incomplete. In this case, the ratio between compounds E and EI depends on the duration (dose) of irradiation: increasing the dose results in an increase in the EI/E ratio. The change in the EI content in a 10^{-6} *M* solution of NHRP is shown in Fig. 4, where the range of dynamic stability of this compound is clearly seen (*D* is *ca.* 90 to 250 Gy).

It should be noted that after radiolysis of HRP significant post-radiation effects exist, mainly related to the reverse EI $\rightarrow$ E transition. Even 20 h after irradiation this process remains incomplete.

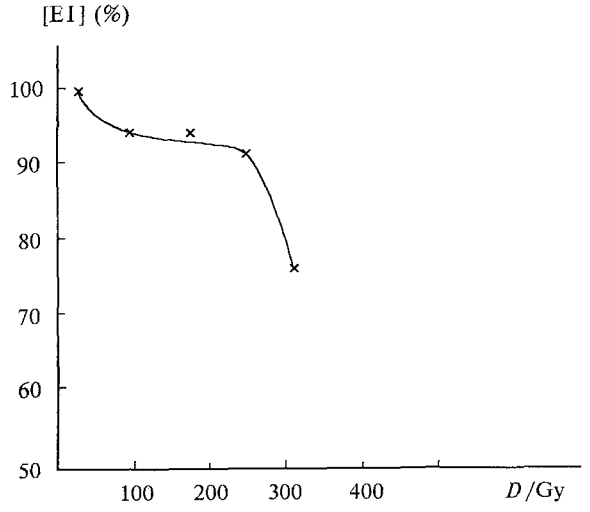

Fig. 4. Dependence of changes in the amount of the EI compound on the irradiation dose (*D*) during radiation inactivation of NHRP (10^{-6} mol L^{-1}) in Tris—HCl buffer (pH 8.0).

Using the method of the second derivatives of the UV spectra, we demonstrated that in a 10^{-6} *M* solution of NHRP there is post-radiation damage of the bond between hemin and protein. The damage correlates directly with the irradiation dose. Increasing the concentration of the enzyme significantly impedes this process.

Using the data[10] on the radiation-chemical yield of H_2O_2 in the radiolysis of water, one can estimate that at $D = 30$ Gy ($t_{irr} = 10$ min) no more than 10^{-7} mol L^{-1} H_2O_2 was formed (in comparison with $5 \cdot 10^{-3}$ mol L^{-1} H_2O_2 during inactivation in the course of the reaction). It remains unclear, why during radiation inactivation this small amount of H_2O_2 formed in the radiolysis of water is able to transform 10^{-6} mol L^{-1} of NHRP into the EI compound. This apparently indicates that either the buffer solutions used are also involved in the formation of additional amounts of H_2O_2, or the EI compound can arise as the result of action of other radiation-induced particles, not only H_2O_2.

The data on the changes in the activity of HRP measured in relation to different substrates during incubation with H_2O_2 and during radiation inactivation of the enzyme reflect the similarity of these processes in the initial stage, especially for NHRP (see Figs. 1 and 2), which can be seen in the existence of the induction period and the formation of the EI compound. The inactivation of HRP by H_2O_2, like radiation inactivation, may be due to a significant contribution by superoxide radicals ($O_2^{\cdot -}$) to this process to modify the inside of the active site of the enzyme. One would expect that this process would result in the total inactivation of the enzyme more effectively than the action of the radiation-induced radicals generated outside the active site. Indeed, this fact was confirmed by our data (see Figs. 1 and 2).

A principal distinction exists between inactivation with H_2O_2 and inactivation through irradiation for RHRP, because in this case the formation of a so-called "molten globule" state is assumed,[5] which is stable in the 0.5—30(45) Gy dose range (see Fig. 2).

Thus, the observed similarity between radiation inactivation and inactivation with H_2O_2 for HRP in combination with the distinctions between the enzymatic activities of NHRP and RHRP measured in relation to different substrates apparently attests to the radical character of the damage in the initial stage of inactivation in both cases. During 10 min of incubation with H_2O_2, the main contribution to inactivation may be due to the generation of superoxide radicals, which modify amino acid residues inside the active site. This modification has the strongest effect on the activity in relation to *o*-phenylenediamine (see Fig. 1). In accordance with the NMR data, the Phe142 and Phe143 residues located at the entrance to the active site are involved in binding aromatic substrates (see Ref. 9). Possibly, the superoxide radicals generated in the oxidation of the peroxide by the EI compound (see Scheme), interact primarily with

these residues, and then with the residues located near the δ-*meso*-ring of hemin, which are responsible for interaction with the iodide ion (see Ref. 11).

Thus, the method of radiation inactivation may be applied both for elucidation of the conformational peculiarities of a biomolecule and for modeling the radical processes that, in particular, result in inactivation of HRP in the course of a reaction.

Experimental

Ammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), *o*-phenylenediamine, isopropyl-β-D-thiogalactopyranoside, sodium dodecylsulfate, trimethylaminomethane (Tris), oxidized glutathione, diiodothreitol, hemin (Sigma, USA), bactotryptone, and yeast extract (Difco) were used. The original pSA261 plasmide with the HRP gene under the control of a tac-promotor was kindly supplied by Amersham International plc. (UK).

The measurements of the peroxidase activities were carried out with a Shimadzu UV 120-02 spectrophotometer at 25 °C in accordance with 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 0.1 *M* NaOAc buffer (2 mL, 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 assumed to be 36800 L mol^{-1} cm^{-1} (Ref. 12). The activities were represented in *E* units (μmol min^{-1}) per 1 mg of protein.

For KI. A KI solution (0.05 mL, 20 mmol L^{-1}) and an aliquot of the enzyme were added to 0.1 *M* NaOAc buffer (2 mL, pH 5.0); the reaction was initiated by the addition of 0.5 % H_2O_2 (0.1 mL). The increase in absorbance was measured at 350 nm, and the extinction coefficient was assumed to be 26000 L mol^{-1} cm^{-1} (Ref. 13).

The concentration of H_2O_2 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 the batch.

Inactivation of the enzymes (at 10^{-7}—10^{-6} mol L^{-1} concentrations) with H_2O_2 was carried out by incubation in the presence of 5 mmol L^{-1} of H_2O_2 in a Tris—HCl buffer (pH 8.0) at 25 °C. Aliquots were taken and the enzymatic activities were measured for the different donor substrates.

The recombinant enzyme was prepared using the procedure described previously.[5] The aqueous solutions of HRP (10^{-7}—10^{-6} mol L^{-1}) were irradiated with a γ-source with P_γ = 0.05 Gy s^{-1}. Then the enzymatic activities in relation to the different substrates were measured taking into account spontaneous and post-radiation inactivations.

The protein content was measured by spectrophotometry.[14] Homogeneity of the enzymatic preparations was tested by electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate.

The spectrum of NHRP and its second derivative were obtained with a Shimadzu 265 FW spectrophotometer. The measurements were performed at 20 °C in the λ = 300 to 750 nm range (see Ref. 7).

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