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Comparative study of horseradish mutant forms by radioenzymology

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Radioenzymology was used to study the recombinant and mutant forms of horseradish peroxidase, namely, F221W, Q176E, Q176A, S35K, E64P, E64S, S35A, and S35KQ176E. Both removal of the Trp residue and introduction of an additional one result in a simpler dose response; the insertion of polar residues stabilizes the enzyme molecule through realization of a more closed conformation. The greatest oscillation changes were found for the replacement by Ala. It was assumed that the binding site of guaiacol as a substrate is located near the residue 64, which is structurally related to the residue 176 and the heme. A scheme of formation of the intermediate through rotation of the Trp aromatic ring was proposed.

Key words: horseradish peroxidase, recombinant horseradish peroxidase, mutant forms of horseradish peroxidase, F221W, Q176A, Q176E, S35A, S35K, E64P, E64S, S35KQ176E.

The radioenzymatic technique implies the use of radiation-induced inactivation of enzymes in combination with kinetic methods for the study of enzymes; in some cases, this extends the scope of investigations of structural and catalytic features of enzymes and enzymatic systems.[1,2] This technique can be combined with genetic engineering, which allows the construction of mutant molecules. Since the effect of ionizing radiation on enzymes is indirect, the irradiated surface area of a biomolecule[3] and the accessibility of amino-acid residues (*i.e.*, the factors characterizing the conformational state of the molecule in the given solution) determine the pattern of dose response (dependence of the catalytic activity on the radiation dose). Therefore, the given method is among the most sensitive (together with tritium planigraphy and X-ray spectroscopy) to the change in the molecule conformation. In particular, it was used to prove the conformational differences between the native and recombinant horseradish peroxidases (HRP and rHRP, respectively)[4] and to elucidate more precisely the role of some residues (by point replacements F41H, W117F, and F143E) in the catalytic cycle.[5—7] The conclusions are based on the analysis of dose responses and radiation-chemical parameters, including G_{in}, G_{act}, the radiation-related yields of inactivation and activation, respectively; D_{80}, D_{50}, D_{37}, *i.e.*, the doses that corre-

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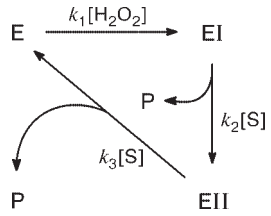
spond to the retention of 80, 50, and 37% of the initial enzymatic activity, respectively; D_{lim}, *i.e.*, the limiting dose after which only inactivation takes place, D_{ind}, *i.e.*, the dose up to which the induction period lasts, *etc.*[8] Each parameter characterizes a section of the dose curve and, correspondingly, the stage (and/or the rate) of changes in the molecule.[2,9,10]

The catalytic cycle (Scheme 1) of horseradish peroxidase (HRP) includes the formation of the so-called Compound I (EI) and Compound II (EII) from the native enzyme (E). The native enzyme (E), EI, and EII can be easily distinguished by spectroscopy when they react with the substrate (S). Previously,[11] it was shown by UV spectroscopy that radiolysis of HRP affords only Compound II.

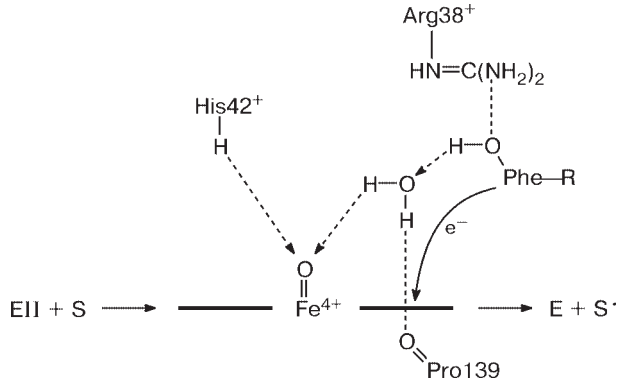
Scheme 1



S is the substrate, P is the single-electron oxidation product

Taking into account the crystal structure of plant peroxidases[12—14] and the ternary complex formed by the enzyme with cyanide and ferulic acid,[15] one can confidently conclude that the cleavage of hydrogen peroxide follows a molecular mechanism and that the water molecule detected in the active site plays an important role in the electron and proton transfer (Scheme 2). This may have significant consequences as regards the damage of the molecule active site induced by the indirect influence of the radiation.

Scheme 2



Mention should be made of the tremendous role of the His residues and the possibility of hydrogen bonding in the protonation—deprotonation catalysis, which was demonstrated for the His42 and Arg38 residues, respectively.[15] Since the role of His in the catalysis is conditioned, among other factors, by its function as a base, the replacement of this residue by Glu proved to be useful for the understanding of specific features of the catalytic cycle. The Glu residue was shown to be displaced in space (from the optimal position of His in the complex) relative to the heme; this violates the structure of the heme—H_2O_2—distal His(Glu) complex, *i.e.*, the ability of the distal residue to form hydrogen bonds.[16] In the H42E mutant, k_1 and k_3 mainly decrease, whereas k_2 is almost insensitive to the replacement of the distal His by Glu. This points indirectly to different roles of proton transfer synchronization at different stages of peroxidase catalysis.

A point mutation usually changes the molecule conformation (in particular, the surface accessible to the solvent); often, it also changes the catalytic properties. In addition, the residues remote from the heme and its immediate environment (for example, Trp117 in HRP[6]) can be of fundamental importance for maintaining the conformation and for exhibiting definite catalytic properties.

In this study, we used mutant forms of HRP to evaluate some conformational features of the enzyme and the position of the substrate-binding sites. The choice of particular mutants was due to different reasons. The replacement F221W provides the possibility of electron donation to the porphyrin ring;[17] an extra Trp residue appears, which does not participate in the catalytic cycle but is rather sensitive to radiation. The Glu64 residue participates in the binding of calcium[18] and maintains the acid-base balance. Therefore, the replacement of this group by neutral proline and serine residues, unable to perform this function, may have a crucial influence on the molecular properties. The replacements Q176E, Q176A, S35K, S35A, and Q176E/S35K (S35KQ176E) provide the possibility of charge variation (redistribution) in the region of hemin propionates, *i.e.*, in the region close to the entrance to the heme-binding cavity. Substrates with different substrate-binding sites were used in the work.

Results and Discussion

Figures 1—6 present the dose response (dependence of the catalytic activity toward two substrates, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and guaiacol, on the γ-radiation absorbed dose) of the mutant HRP in comparison with the wild-type recombinant form (rHRP).[19] The variation of the activity under inac-

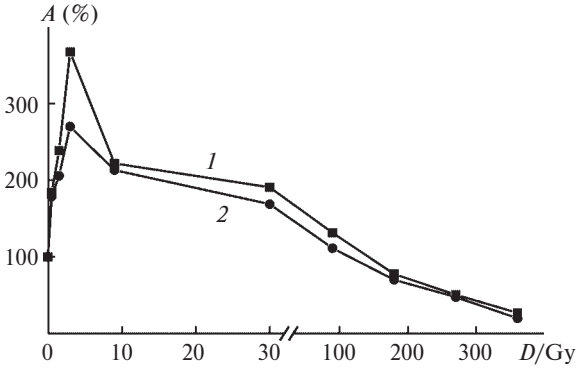


Fig. 1. Dose response of 10^{-7} *M* rHRP (Tris-HCl, pH 7.0); substrates: ABTS (*1*), guaiacol (*2*).

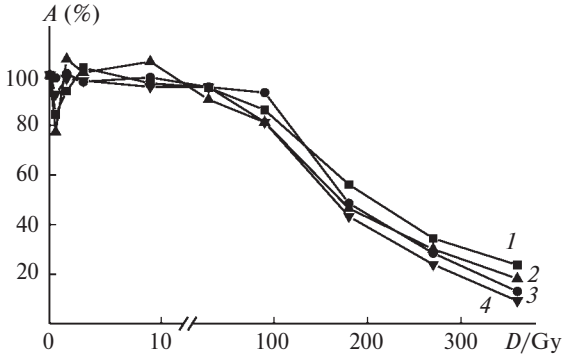


Fig. 2. Dose response of 10^{-7} *M* F221W (*1*, *2*) and W117F (*3*, *4*)[4] (Tris-HCl, pH 7.0); substrates: ABTS (*1*, *3*), guaiacol (*2*, *4*).

tivation conditions, which is described by a nearly exponential curve, is characterized conveniently by doses corresponding to the retention of 80% (D_{80}) and 50% (D_{50}) of the enzyme catalytic activity. These doses give an idea of the stability under conditions where conformational and mixed (conformational and destructive) processes, respectively, predominate in the enzyme upon irradiation.[2] However, in the case of more complex patterns of

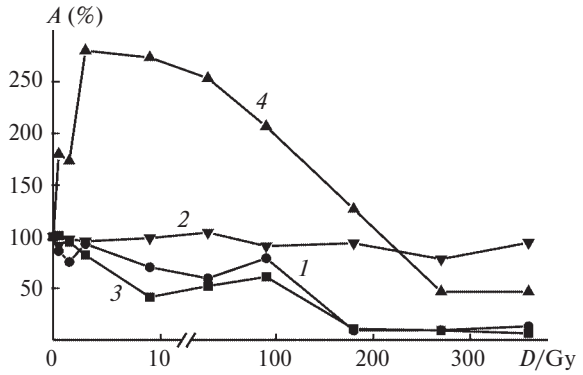


Fig. 3. Dose response of 10^{-7} *M* E64S (*1*, *2*) and 10^{-6} *M* E64P (*3*, *4*) (Tris-HCl); substrates: ABTS (*1*, *3*), guaiacol (*2*, *4*).

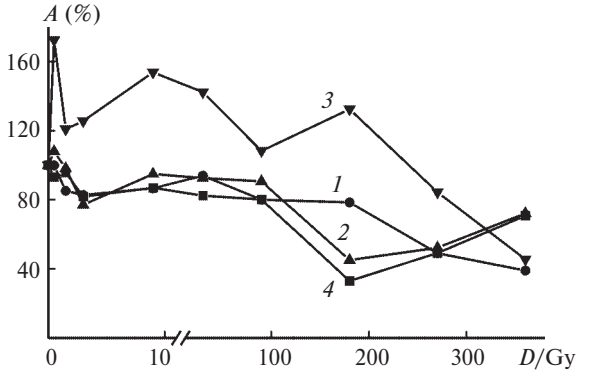


Fig. 4. Dose response of 10^{-7} *M* Q176A (*1*, *2*) and Q176E (*3*, *4*) (Tris-HCl, pH 7.0); substrates: ABTS (*1*, *3*), guaiacol (*2*, *4*).

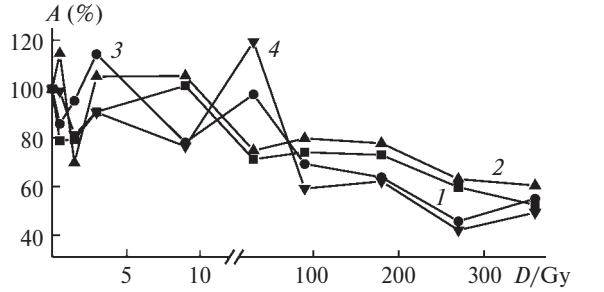


Fig. 5. Dose response of 10^{-7} *M* S35K (*1*, *2*) and S35A (*3*, *4*) (Tris-HCl, pH 7.0); substrates: ABTS (*1*, *3*), guaiacol (*2*, *4*).

dependence of the catalytic activity on the dose, the notion of stability becomes less clear because other characteristics (for example, D_{lim}) appear due to superposition of numerous processes. Therefore, the notion of stability is conventional, and different radiation-related parameters characterize different processes.

Table 1 presents the radiation-chemical parameters of the mutant HRP, which characterize both the differences in the stability and properties of these forms caused by the substitutions made and the difference from the initial rHRP.

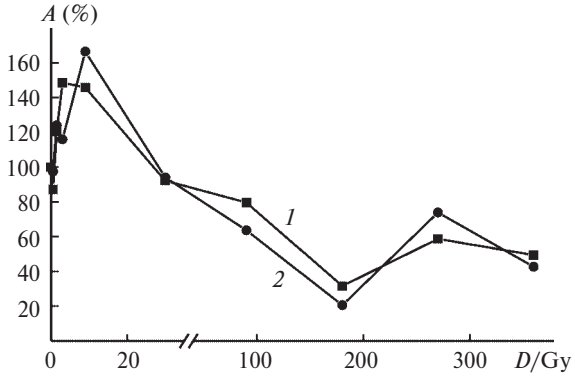


Fig. 6. Dose response of 10^{-7} *M* QESK double mutant (Tris-HCl, pH 7.0); substrates: ABTS (*1*), guaiacol (*2*).

Table 1. Radiation-chemical parameters of the HRP mutant forms ($1 \cdot 10^{-7}$ mol L^{-1}, 50 m*M* Tris-HCl buffer, pH 7.0)

HRP form	Process scheme[a]	Substrate	D_{lim}	D_{act}	D_{ind}	D_{in}	$D_{plateau}$	Δ_{in}	Δ_{act}[b]
			Gy					%	
rHRP	a—in	ABTS	3	3	—	—	—	265	—
	a—in	Guaiacol	3	3	—	—	—	170	—
Q176E	ind—in—a—pl—in	ABTS	9	9	1.5	3	9—90	20	25
	ind—in—pl—in	Guaiacol	90[c]	—	1.5	3	3—90	—	20
Q176A	a—in—a—in	ABTS	180	0.5, 9	—	1.5	40—180	75, 30	50
	in—pl—in	Guaiacol	180	—	—	1.5	1.5—180	—	20
S35K	in—a—in	ABTS	180	9	—	0.5, 30	30—180	25	25, 30
	a—in—a—in	Guaiacol	180	0.5, 3	—	1.5, 30	30—180	16, 40	45, 30
S35A	in—a—in—pl—in	ABTS	30	3, 30	—	0.5, 9	90—180	30, 20	18, 42
	in—a—in	Guaiacol	30	30	—	9	—	45	25
E64P[d]	ind—in	ABTS	90	—	—	9	9—30	—	60
	a—pl—in	Guaiacol	30	3	—	9	3—30	180	—
E64S	ind—in	ABTS	90	3	—	1.5	9—30	—	25
	ind—in	Guaiacol	180	—	180	—	—	—	—
S25KQ176E	in—a—in	ABTS	3	3	—	0.5	—	60	20
	a—in—a—in	Guaiacol	270	9, 270	—	180	—	66, 60	146
F221W	in—a—pl—in	ABTS	30	3	—	0.5	9—30	16	16
	in—a—in	Guaiacol	9	3	—	0.5	—	30	23
W117F	ind—in	ABTS	90	—	90	—	—	—	—
	ind—in	Guaiacol	30	—	30	—	—	—	—

[a] The following designations are used: in is inactivation, a is activation, pl is plateau, ind is induction period.
[b] The activation magnitude was calculated in all cases as the difference between the subsequent and preceding catalytic activities.
[c] This D_{lim} value does not take into account activation at high doses, which is discussed in the paper.
[d] The concentration is $1 \cdot 10^{-6}$ mol L^{-1}.

The curves for the initial recombinant enzyme (see Fig. 1) exhibit a large activation peak (for both substrates) and approximately equally high total stability for the two substrates (D_{50} = 270 Gy). However, the dose of the onset of inactivation is relatively low (D_{lim} = 3 Gy).

Comparison of the dose responses of double-tryptophan (F221W) and tryptophan-less W117F [6] mutants (see Fig. 2) shows that the dose responses of F221W for two substrates with primary inactivation at D = 0.5 Gy virtually coincide but differ appreciably from the dose response of rHRP. This attests to a different conformation of the given mutant, which is manifested as a "simpler" dose response both for the tryptophan-less form and for the mutant with two Trp residues. The overall stabilities of these two mutant forms are lower than that of the recombinant enzyme; however, the D_{lim} value somewhat increases (see Table 1). A substantial increase in D_{lim} (up to 90 Gy) was found in the case of the W117F mutant, while the overall stability is similar to that of F221W. According to our views on the role of Trp residues, the introduction of the additional residue not involved in the catalytic cycle should increase the mutant stability by decreasing the probability of effects caused by low radiation doses. This is actually the case for the D_{lim} and D_{80} values but D_{50} somewhat decreases. The behaviors of the two mutants toward different substrates are slightly different. Thus, both the removal of the sole Trp residue and the introduction of an additional Trp residue sharply decrease the contribution of processes resulting in activation. The overall dose responses of these mutants are simpler; however, some "subtle" effects are observed for F221W (for low doses). Previously,[20] in a study of angiotensin-converting enzyme (ACE), the protective role of Trp residues was demonstrated under conditions where some of them did not participate in the catalytic process (self-protector mechanism). In HRP, the appearance of an additional Trp residue due to the replacement of Phe, like the disappearance of the only residue (W117F), entails a smoother dose dependence with a substantial increase in the period of latent changes.

The Glu64 residue, which is involved in calcium binding and can behave similarly to the His residues,[16] was replaced by the polar Ser and Pro residues, which are prone to form hydrogen bonds. For the E64S mutant, no large activation peak is observed in the dose response (see Fig. 3), which may imply a more rigid structure of the active site compared to that of rHRP (thus in the case of ACE, activation predominated when the flexibility of the active site increased[21]). This has the most pronounced influence on the guaiacol-binding site because the dose response with respect to this substrate acquires

a persisting stability against latent disturbances (D_{lim} = D_{ind} = 180 Gy). Slight "subtle" effects are observed toward ABTS.

The replacement by the Pro residue (E64P) decreases the stability as regards ABTS and has only a slight effect on the guaiacol-binding site and its surrounding, because the dose response has an intense activation peak, which is observed at the same dose as for rHRP but is much broader. High activation is also observed for higher concentrations of the enzyme ($1 \cdot 10^{-6}$ mol L^{-1}) where aggregation processes have started and, as a consequence, the possibility of variation of the conformational state of the molecule incorporated in an aggregate is limited by the presence of additional bonds. Previously,[21] activation was observed for ACE with a decrease in the enzyme concentration, *i.e.*, where the ratio of the amount of the active products of water radiolysis to the number of enzyme molecules increased. For the E64S mutant and ABTS, the increase in the concentration from $1 \cdot 10^{-7}$ (see Fig. 3) to $1 \cdot 10^{-6}$ mol L^{-1} eliminated the activation and stabilized the molecule ($D_{50} > 360$ Gy). The fact that activation of E64P takes place at a higher enzyme concentration attests, apparently, to a rather high flexibility of the guaiacol-binding site and the active site of this mutant. We suggest that the guaiacol-binding site, which should be located near the heme, according to previously published data,[22] is situated also near the residue 64.

The replacement of the noncharged polar residue Gln176 by the negatively charged Glu residue (Q176E) and by the nonpolar Ala residue (Q176A) was also considered (see Fig. 4). The dose response of the Q176E mutant exhibits no activation peak and no distinctions for different substrates. After slight inactivation, an intermediate is formed, which is stable for doses of 9 to 90 Gy. It is of particular interest that at high doses ($D > D_{50}$) where unfolding and destruction processes predominate,[2] the change in the accessible surface area of the globule caused by these processes gives rise to a conformational state able to undergo secondary activation toward both substrates (see Fig. 4). Evidently, this points to a decrease in the accessible surface area (and, hence, in the globule size) in Q176E relative to rHRP, which might be due to the formation of additional bonds by the Glu residue. It should be noted that the F143E mutant, which we have studied previously,[5] showed the formation of a similar intermediate for doses of 9—90 Gy, while its behavior in the region of <9 Gy was different. However, as the radiation dose increased, the catalytic activity of F143E slowly decreased. It should be emphasized that different forms of the enzyme produce intermediates at different doses, *i.e.*, the initial packing densities of molecules are different. The D_{lim} values for Q176E (90 Gy) are higher than those for rHRP; however, the overall stability decreases ($D_{50} < 180$ Gy).

The introduction of a rather inert Ala residue (see Figs. 4 and 5) has a lesser effect on the accessibility of the ABTS-binding site than other substitutions, thus increasing the stability of Q176A with respect to this substrate ($D_{80} = 250$ Gy, $D_{lim} = 180$ Gy). However, primary inactivation giving a radiation-stable intermediate is observed when guaiacol is used. The total stability (D_{50}) does not change. If our assumption concerning the position of this site near residue 64 is true, the replacement Q176A should disrupt the structural interconnectivity of this region. Generally, the replacement by Ala is seen to increase the possibility of conformational instability, thus promoting higher sensitivity of the molecule to the action of water radiolysis products at lower doses.

The appearance of an extra positive charge upon the replacement of the polar Ser residue (S35K) entails slight effects at "low" doses, whose amplitude and period vary, depending on the substrate. When the dose is increased above 180 Gy, smooth inactivation occurs (see Fig. 5). These effects are more pronounced toward guaiacol. The effects observed toward ABTS are enhanced when the Ser35 residue is replaced by nonpolar Ala (S35A), whereas in the case of guaiacol, activation at a higher radiation dose ($D_{act} = 30$ Gy) appears in this case. It seems that the distance between the peaks increases and the first of them should appear at even lower doses, *i.e.*, the effects are also possible at doses lower than those considered. In addition, in the presence of clear-cut activation, the stability to latent damages appears to decrease. The overall stability and the D_{lim} values remain high (180 and 360 Gy, respectively).

Now we consider the double mutant, S35KQ176E, in which the appearance of the negative charge on a globule (Glu176) is counterbalanced by introduction of the positive charge (Lys35). Its dose response is shown in Fig. 6. The double replacement entails an increase in activation, which shifts along the dose vector upon replacement of the substrate. The degree of activation barely depends on the substrate. The dose response of the double mutant resembles most closely that for rHRP. In addition, for doses of up to 30 Gy, its dose response is more similar to that for the S35K mutant; conversely, for higher doses, it resembles the Q176E mutant characterized by activation at high doses (270 Gy).

Presumably, the initial enzyme molecule has a set of allowable conformational states, which occur in equilibrium. Any variation changes the conformational equilibrium, which can either return to the initial position or shift toward one of the states, in particular, toward a highly active state. In this case, the conformational equilibrium of the double mutant is closer to that of the initial rHRP than these equilibria of the forms containing additional positive or negative charges. Thus, the ability of the active site of the horseradish peroxidase to fix the conformational state capable of being highly acti-

vated depends on the charge of the amino acid residues near the active site.

The ABTS-binding site is known to interact with the active site along an electron transport chain; therefore, the activity depends on the range and disposition of the amino-acid residues that accomplish the electron transport,[4,8] *i.e.*, on their accessibility. This is why the activation observed in the initial rHRP enzyme cannot be manifested for any mutant toward ABTS. The form whose dose response approaches most closely that of the initial enzyme is the double mutant in which the globule bears no uncompensated charge capable of creating either hindered or, conversely, preferential conditions for the penetration and interaction with the enzyme of any product of water radiolysis or any other active product. For other enzyme forms, slight activation—inactivation effects took place; in some cases, they can be regarded as oscillations with different amplitudes and periods. Depending on the enzyme form, the effects shifted along the dose vector.

The features of the guaiacol-binding site are unknown; it has only been established that it is located near the heme. A conformation (accessibility) of the surrounding of the substrate-binding site for this substrate resembling most closely the initial rHRP was found for the E64P mutant and for the Q176ES35K double mutant. The obtained data suggest that the guaiacol-binding site is located near the residue 64 and depends on the extent of exposure of the residue 176, because in the Q176E and Q176A mutants, activation and oscillations in the dose response for this substrate completely disappear. Thus, one may assume a structural interrelation of the residues 64 and 176 and the heme in the horseradish peroxidase molecule (at least, in the recombinant form).

Apparently, substantial changes of the conformational equilibrium take place on the removal of the only Trp residue, which increases the stability and gives rise to a smoother curve of dose response. The appearance of polar residues stabilizes the enzyme molecule, evidently, due to the formation of hydrogen bonds and realization of a more "closed" structure in this region of the molecule. The replacement by Ala gives rise to the greatest oscillation phenomena, *i.e.*, this replacement hampers the fixation of one conformational state.

Virtually all rHRP forms produce intermediates more or less stable against radiation, indicating that the unfolding proceeds through at least one fixed structural state formed under the action of a radical impact. It is worthy of note that this state has not been detected on treatment with hydrogen peroxide.[11] Thus, the formation of this transient state does not require a change in the heme itself, some set of point damages in the polypeptide chain being sufficient. The fact that this intermediate was not detected for the tryptophan-free mutant, but a long lag period followed by the classical exponential inactivation was observed instead,[6] suggests that this state is related to modification (or a change in the accessibility) of the only Trp residue. In our opinion, this might be rotation of the aromatic ring plane due to the formation of the Trp—OH$^{\bullet}$ type radical (similar to the known aromatic radical formed upon an attack of tryptophan by tritium atom[23]) induced by an attack of the most efficient product of water radiolysis, *viz.*, $^{\bullet}$OH radical.

Experimental

Mutant HRP forms provided by Japanese colleagues M. Tanaka and I. Morishima (Kyoto) and wild-type rHRP were used in the study. The method for their preparation was described previously.[17—19,24] The *RZ* value (the ratio of the optical density values in the electronic spectrum of the enzyme at 403 and 280 nm characterizing the saturation of the specimen with hemin, *i.e.*, the specimen purity) was 2.8—3.3, the specific activity of rHRP was 4000 U mg^{-1}, that of mutants was ~600 U mg^{-1}.

The catalytic activity toward ABTS (Sigma) was measured as follows: 0.05 mL of an aqueous solution of ABTS (8 mg mL^{-1}) and ~20 μL of the enzyme solution under study (in Tris-HCl, pH 7.0) was added to 2 mL of 0.1 *M* sodium acetate buffer (pH 5.0), and the reaction was initiated by adding 0.1 mL of H_2O_2 (100 mmol L^{-1}). The molar extinction of the ABTS oxidation product was 36800 L mol^{-1} cm^{-1} at 405 nm.[25]

The catalytic activity toward guaiacol (Sigma) was measured as follows: 0.15 mL of an aqueous solution of guaiacol (1 mg mL^{-1}) and ~10 μL of the enzyme solution under study (in Tris-HCl, pH 7.0) was added to 0.2 mL of 0.1 *M* sodium acetate buffer (pH 5.0) and the reaction was initiated by adding 0.1 mL of H_2O_2 (100 mmol L^{-1}). The molar extinction of the guaiacol oxidation product was 25500 L mol^{-1} cm^{-1} at 436 nm.[26]

Solutions of enzymes (10^{-7} *M*, 0.2 mL, Tris-HCl buffer, pH 7.0) were irradiated using a γ-source (^{137}Cs) with a dose rate of 0.05 Gy s^{-1}.

The experimental error was estimated as the confident inaccuracy of the results corresponding to 95% confidence probability and found for both the change in the catalytic activity in one irradiation experiment (not less than three measurements) and for different irradiation experiments (not less than two experiments).

The catalytic activity was measured 1 h after irradiation, with respect to the initial, nonirradiated enzyme; in the vast majority of experiments, this period of time was sufficient for secondary reactions to take place (including the action of H_2O_2) and no postradiation effects were observed. Otherwise, the curve of postradiation effects up to saturation was constructed, and this was taken into account for each particular radiation dose.

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