Reviews

The use of a radiation inactivation method for characterization of the structure and catalytic properties of enzymes

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The use of a radiation inactivation method for studies of the properties of enzymes of different classes is discussed. The possibilities of characterization of enzymes on the basis of the radiation-chemical parameters concerned are described.

Key words: radiation-induced inactivation, angiotensin-converting enzyme, horseradish peroxidase, chymotrypsin, subtilisin-72, substrate specificity, radiation-chemical yield of inactivation.

The radiation inactivation (RI) method has been used in enzymology for a long time.[1] It is based on the target theory[2] and analysis of complex equilibrium systems.[3,4] The main parameters of the process are the D_{37} value (the dose at which 37% of the initial activity is retained), derived from the plot of the effect (inactivation) *vs.* the absorbed dose (if this curve is exponential), and the values characterizing the equilibrium between the components or subunits of the system under study. However, it is known that dose dependences are exponential only in a narrow range of concentrations of enzymes,[5,6] and in most cases, they are expressed by uneven curves. This phenomenon is evidently observed for both enzymic systems and individual enzymes, and the shape of the curve depends on the $[R]/[E]_0$ ratio, where [R] is the number of radicals formed and $[E]_0$ is the initial concentration of the enzyme.[6] The exponential dose dependence and the D_{37} value make it possible to determine the size of a molecule (molecular weight), but only provide a little information on the structure and the mode of action, as well as on the conformational changes in the process of inactivation of the enzyme. The latter can be revealed in those cases where the dose curves have uneven shapes.[5] Analyses of these unsmooth curves in order to characterize the structure and properties of the enzymes have been performed for papain,[5] α-chymotrypsin,[6–12] subtilisins,[13–16] angiotensin-converting enzyme (ACE),[17,18] and horseradish peroxidase C (HRP).[19–24] These enzymes belong to different classes and differ in the complexity of their structures, the mechanism of catalysis, and physiological function. Chymotrypsin is a thoroughly studied enzyme,[25] while the information on ACE is still insufficient despite its extensive investigation. Chymotrypsin and subtilisins are serine proteinases, and ACE and HRP[26] are metal-containing glycoproteins and are hydrolase and oxidoreductase, respectively. Thus, the range of the activity pattern of the enzymes studied is quite broad.

In the case of aqueous solutions, RI is caused by the indirect action of radiation due to the formation of active products in radiolysis of water,[27] which can react with the enzyme by acting on the exposed (accessible)

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sites of the molecule. Hence, radiation-induced modification of the enzyme depends on its structure, conformation, and catalytic properties (*i.e.*, localization of the substrate-binding site). The study of the response of a molecule to radiation provides information on these peculiarities of the enzyme.

It was shown[6] that any dose curve, independent of its shape, can be divided into three parts according to the predominance of the damages incurred. In the first step, almost up to D_{80} (the dose, at which 80% of the enzyme activity is retained), conformational changes occur. Numerous experiments[6,7,17,28,29] demonstrated that Trp and Tyr residues are the most important in this step. If the percentage and absolute number of these residues are high, some of them would play a protective role, thus increasing the radiation stability of the enzyme. When carrying out RI, the exposure of these residues will change depending not only on the dose but also on the irradiation conditions; this can characterize the effect of the conditions on the conformational flexibility of the active center, which evidently depends on those Trp and Tyr residues that are directly connected with the structure of the active center. Determination of the number of these amino acid residues (more exactly, the change in it) can be performed by the method of second derivatives of UV spectra,[17,22] by fluorimetry,[7,28,29] and by the method of amino acid analysis (Tyr).[7,14] For example, the change in the number and accessibility of the Trp and Tyr residues in ACE juxtaposed to the change in the catalytic activity during irradiation at different pH was found using the method of second derivatives of UV spectra[17,18] (Fig. 1). Later, this observation made it possible to elucidate the role of these residues in the structure of the enzyme. In the case of chymotrypsin and subtilisin-72[14] (Fig. 2), amino acid analysis provides information on the radiation sensitivity of various amino acid residues. It should be noted that both these methods clearly demonstrate the importance of the Trp and Tyr residues for two very different objects such as ACE and subtilisin.

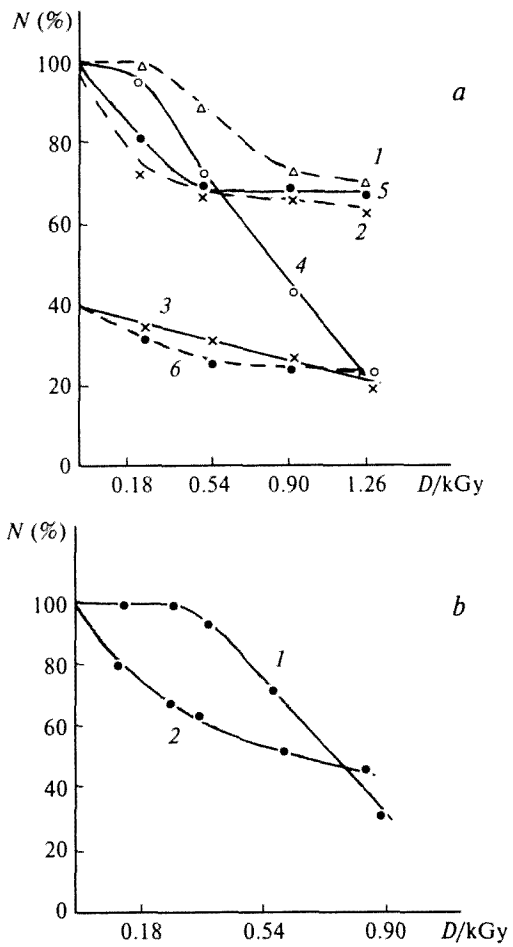

Fig. 1. Transformations of ACE under the effect of radiation: *a*. 10^{-6} *M* solution of ACE (0.15 mol L^{-1} of NaCl) at pH 6.5 (*1—3*) and 8.5 (*4—6*). Curves *1* and *4*: change in enzyme activity *vs.* radiation dose; *2* and *5*: destruction of the Trp and Tyr residues (analysis by the method of second derivatives of UV spectra); *3* and *6*: exposure of Trp residues in the molecule of ACE for the solvent; *Z*-PheHisLeu was used as a substrate.[18] *b*. 10^{-6} *M* solution of ACE (0.15 mol L^{-1} of NaCl), pH 7.5, furylacryloyl-PheGlyGly was used as a substrate: *1*: change in enzyme activity, *2*: change in intensity of Trp and Tyr peaks in the second derivative of UV spectra.[17]

The first part of the dose curve is the most informative, especially in the range of "low" doses (*i.e.*, the doses, which can be assumed as low for objects *in vitro*). For the proteases studied, only inactivation was observed in this range (from 0.2 Gy).[6] At the origin of the dose curve, the relative decrease in enzyme activity depends only slightly on the initial concentration of the enzyme, while as the doses (time of irradiation) increase, the concentration differences become significant[6] (Fig. 3). For such an enzyme as ACE, in the range of "low" doses alternating processes of activation and inactivation, and the range of stability can be observed.[18] These are conveniently described as D_{act} (the dose at which the activity is maximum), D_{in} (the dose, at which the maximum inactivation is observed if subse-

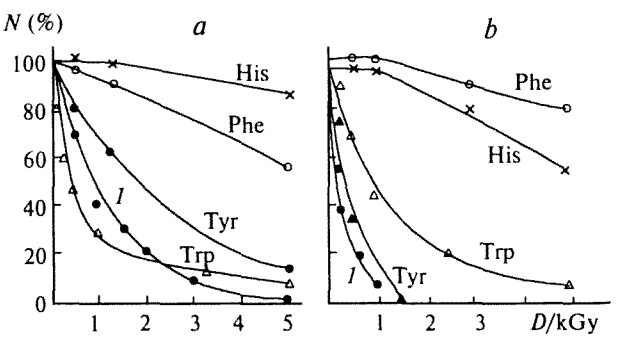

Fig. 2. Change in the number of amino acid residues (*N*) in subtilisin-72 depending on the radiation dose[14] determined by amino acid analysis (His, Phe, Tyr) and fluorimetrically (Trp): *a*: pH 8.5, 0.1 *M* NaCl+NaOH; *b*: pH 5.0, $5 \cdot 10^{-3}$ *M* acetate buffer, *1*: change in enzyme activity.

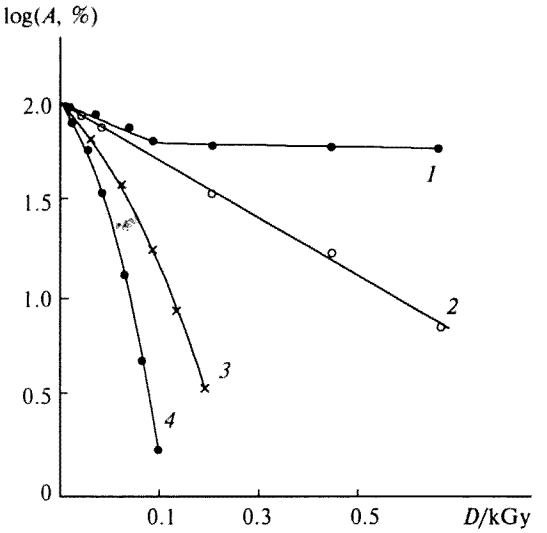

Fig. 3. Semilogarithmic dose dependences of changes in the enzyme activity of α-chymotrypsin:[6] 10^{-4} (*1*); $7.5 \cdot 10^{-6}$ (*2*); 10^{-6} (*3*); 10^{-7} (*4*) mol L^{-1}.

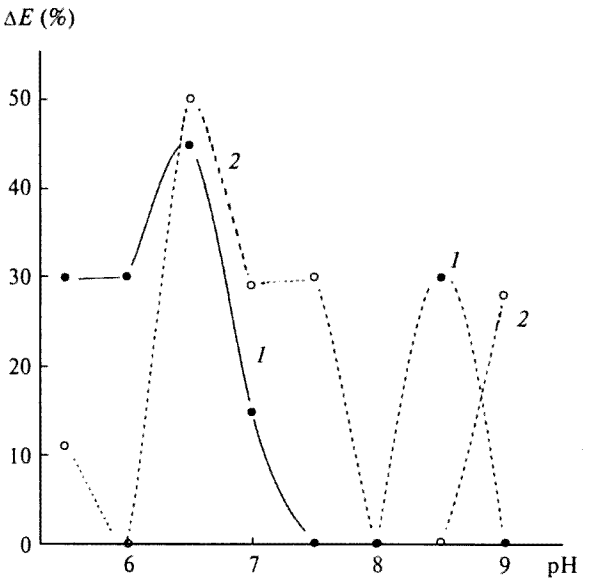

Fig. 4. pH-Dependence of ACE activation (ΔE) (0.15 *M* NaCl, phosphate-borate buffer):[18] 10^{-8} (*1*), 10^{-7} (*2*) mol L^{-1}. Primary activation is designated by the solid line, and secondary activation is designated by the dotted line.

quent irradiation causes an increase in enzyme activity), and D_{plat} (the dose range of stability); the dose (D') after which final inactivation occurs is also very informative, although in some cases, the dependence can be unsmooth at higher doses too.

The data obtained allowed us to assume that the highest activation of the enzymes is observed under conditions in which the conformational flexibility of the active center is maximum. In particular, this is confirmed for ACE.[18] Thus, for an enzyme concentration of 10^{-8} mol L^{-1}, the "primary" activation,* which is maximum at pH 6.5 (Fig. 4, curve *1*), can be seen. The study of ACE by the RI method as a function of the pH and concentration of the enzyme showed that its conformational flexibility is highest just at this pH. This is in agreement with the published data[30] on dissociation of Zn from the molecule of ACE. At the same time, in going to pH 8.5, primary activation was not observed and the character of the changes in the radiation-chemical parameters suggests that the conformation becomes more rigid. An increase in the concentration to 10^{-7} mol L^{-1} (Fig. 4, curve *2*) decreases the $[R]/[E]_0$ value, which causes relative deficiency of radicals and, hence, makes "primary" activation impossible. The competition between different reactions of radicals with the enzyme molecules is potentiated, and therefore, more complex changes in the dose dependences as a function of the pH occur. Here, the processes of activation are manifested as "secondary", *i.e.*, occurring after the lag-period or primary inactivation. However, in this case, the secondary activation maximum also occurs at pH 6.5, and the minimum occurs at pH 8.0—8.5, which is in accordance with the aforementioned changes in conformational flexibility.

When estimating the radiation sensitivity of ACE, one should take into account that it has two active centers in two domains[31,32] and that it is a glycoprotein. Due to the presence of a large number of Trp and Tyr residues (32 and 35, respectively) and carbohydrate residues (~30%), even at doses ≥ 50 Gy, a lag-period characterized by D_{ind} occurs under certain conditions. The autoprotective role of carbohydrates is confirmed by the presence of a lag-period on the dose curve for HRP,[17] which has only one Trp residue and five Tyr residues, but is a glycoprotein like ACE (*ca.* 20% of carbohydrate residues).[26] In addition, no lag-period was observed for irradiation of deglycosylated, recombinant HRP under any of the conditions studied.[20]

The second region of the dose curve is characterized by an increase in the contribution of the stochastic damage of the amino acid residues exposed at this time in according to their radiation sensitivity, which can be approximately estimated by irradiating peptides.[33] This stage of inactivation is best characterized by D_{50} (the dose at which 50% of the enzyme activity is retained). Simultaneously, unfolding of the enzyme molecule continues. The overall rate of these processes can be estimated using the radiation-chemical yield of inactivation per 100 eV of absorbed energy (G_{in}), which is determined from the segment of the curve immediately after a real or arbitrary lag-period (we define the arbitrary lag-period as the dose interval in which alternating inactivation, activation, and stability ranges are ob-

* The activation immediately observed on a dose curve (for initial doses) is defined as "primary". The "secondary" activation takes place after the primary inactivation or the lag-period.

served). In the case of a "simple" enzyme (for example, chymotrypsin), G_{in} is the "rate" of fall of the dose curve determined for different parts of this curve. In the initial part (up to D_{80}), G_{in} characterizes the rate of unfolding of the molecule together with the $G[-(Trp)]$ value, which is assumed[28,29] to be approximately equal to the radiation-chemical yield of unfolded molecules, G_{unf}. In the second part (up to D_{50}), the comparison of G_{in} and $G[-(Trp+Tyr)]$ makes it possible to separate the contributions of conformational and stochastic decomposition. On the whole, an increase in G_{in} indicates higher radiation sensitivity of the molecule. A comparison of G_{in} for several enzymes obtained under the same conditions also allows judging the degree of unfolding of the molecule (*i.e.*, the increase in the accessible surface, S_{acc}). From the initial value of S_{acc},[34] G_{in}, and the amino acid sequence, namely, the localization of Trp and Tyr residues in the enzyme molecule, one can approximate the change in S_{acc} depending on the dose and the most probable sites of damage of the molecule expected in the stochastic stage.

Finally, the third part of the dose curve mainly reflects the decomposition of amino acid residues when tertiary and, in part, secondary structures have already been "spoiled" and can be characterized by D_{37} (the dose at which 37% of the enzyme activity retains).

It seems interesting that the pH-dependence of G_{in} (or k_{in}^{eff}, effective inactivation rate constant) for different enzymes has a sharp maximum at pH 7—7.5. It was observed for ACE,[17] HRP,[20] and trypsin-like enzymes[14] (Fig. 5). Evidently, in this pH range, the conformational transition occurs with the formation of a state with a significantly higher S_{acc} value. Since this transition is observed for enzymes of different classes in the same pH range (with slight differences), it should be assumed that the nature of this phenomenon is the same. Most probably, it is the His residues adjacent to the active center, but not directly bound with heme, if the enzyme is heme-containing (because in the latter case, the pK values for His residues are in the acid area[35]) that are responsible for the transition. Evidently, deprotonation of these residues affects the state of the active center, indicating its accessibility, and for different enzymes, the data on these changes can be different. The properties of this "unfolded" state depend strongly on the presence of a buffer and on the composition of the medium.[17]

An important question in enzymology is the substrate specificity reflecting the catalytic properties of the enzyme in relation to the localization of substrate-binding sites. The RI method can play an important role in their characterization. This study was carried out by us[19,23] for HRP. It was shown that substrates, *viz.*, *o*-phenylenediamine and phenol—antipyrine, have either the same or adjacent substrate-binding sites[21] (Fig. 6), while the iodide ion binds directly to the porphyrin ring of heme, and electron transfer from porphyrin to the iodide anion occurs without participation of the protein component.[20,23] This is in agreement with the data from other methods[36,37] that the iodide anion is a two-electron donor, is oxidized in one step, and it interacts directly with the δ-meso ring of hemin. As for ABTS, its substrate-binding site is near the "entrance" to the active center of the enzyme and its properties depend substantially on the state of the protein component bound with hemin,[22] *i.e.*, ABTS requires a definite arrangement of the heme—protein complex for functioning of the binding site of the substrate. Using the RI method, it was demonstrated that the nature of the peroxidase used (native, *i.e.*, glycosylated, HRP (NHRP), or recombinant, *i.e.*, prepared by molecular engineering (RHRP)) is important. NHRP and RHRP differ not only in the presence or absence of carbohydrates. RHRP was found to be much less active and radiation-stable than NHRP. It is interest-

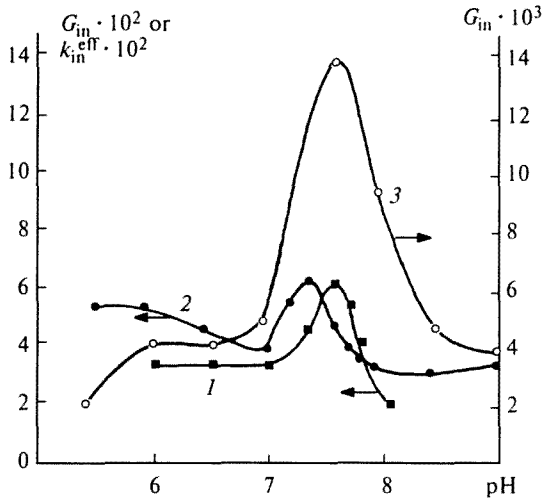

Fig. 5. pH-Dependence of G_{in} (*1* and *3*) and k_{in}^{eff} (*2*): *1*, HRP (10^{-7} mol L^{-1}, substrate: ammonium 2,2′-azino-bis(3-ethylbenzthyazoline-6-sulfonate) (ABTS));[20] *2*, subtilisin-72 (substrate: *N*-acetyl-L-tyrosine ethyl ester);[14] *3*, ACE (10^{-7} mol L^{-1}), 0.025 *M* phosphate-borate buffer, fluorimetric determination, substrate: *Z*-PheHisLeu.[18]

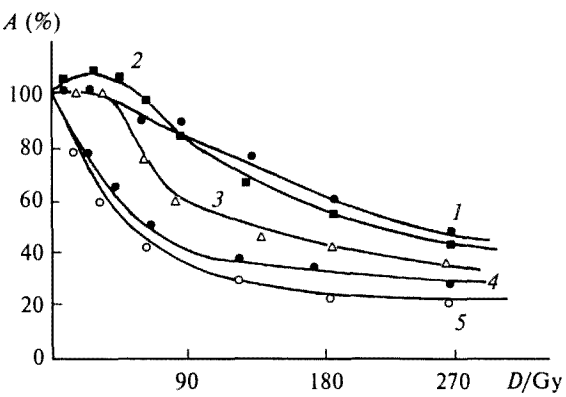

Fig. 6. Radiation inactivation of NHRP (10^{-7} mol L^{-1}, 0.1 *M* Tris-HCl buffer, pH 8.0). Substrates: *1*: KI, *2*: guaiacol, *3*: ABTS, *4*: phenol—antipyrine, *5*: *o*-phenylenediamine.[21]

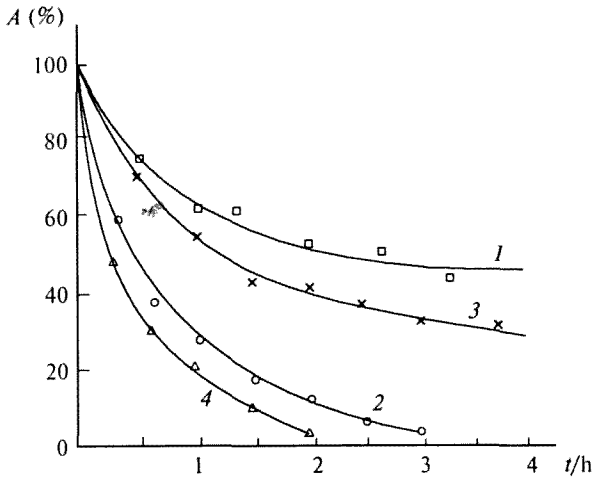

Fig. 7. Thermal inactivation of NHRP (*1* and *2*) and RHRP (*3* and *4*), 10^{-7} mol L^{-1}, phosphate buffer, pH 8.0: *1* and *3*: 50 °C, *2* and *4*: 80 °C (ABTS as a substrate).[20]

ing that an analogous phenomenon was observed under conditions of thermal inactivation (Fig. 7); an increase in the temperature, as well as an increase in the irradiation dose, decreases the difference between NHRP and RHRP. This points to the similarity of thermal and radiation damages.

Thus, the principal differences in these forms of HRP involve the conformation of the heme—protein state and are revealed in the step of introduction of hemin. In addition, inactivation of NHRP during its life cycle (in the course of the reaction), which generates inactive molecules, which can serve as a radical trap, and, hence, act as radioprotectors, should be taken into account.

The formation of the radiation-stable state in the dose range of D_{plat} = 3—45 (in some cases, up to 90) Gy is a characteristic feature of RHRP. Therefore, one can state that the RI method confirms the published data[38] on the state termed "molten globule" through which RHRP passes during unfolding and folding of the molecule. One can conclude that it possesses very high conformational flexibility of heme without perturbation of substrate-binding sites and therefore becomes radiation-stable. To achieve this state, primary inactivation corresponding to a *ca.* 3 Gy dose is required. In light of the facts discussed above, one cannot exclude that this inactvation is related to the single Trp residue in the HRP molecule, although this has not yet been documented. The change in conformation in the transition from NHRP into RHRP alters the accessibility of the substrate-binding sites, making the binding sites for guaiacol and ABTS more accessible and "closing" the substrate-binding sites for *o*-phenylenediamine and phenol—antipyrine, which according to our data[20] are located in the same fragment of the molecule. This conclusion follows from the difference in the dose dependences for NHRP and RHRP with respect to the corresponding substrates (Figs. 6 and 8).

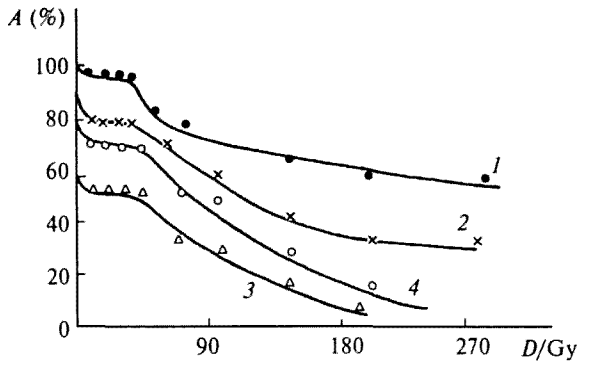

Fig. 8. Radiation inactivation of RHRP (10^{-7} mol L^{-1}, 0.1 *M* Tris-HCl-buffer, pH 8.0); substrates: *1*: KI, *2*: phenol—antipyrine, *3*: guaiacol, *4*: ABTS.

The development of molecular engineering for designing protein molecules makes it possible to use the RI method for a comparative study of mutant forms and provides valuable information on the catalytic properties of an enzyme. The following mutant forms of RHRP were prepared: Phe41→His (F41H) and Phe143→Glu (F143E),[36] which are less active than RHRP. They were studied by the RI method.[19,23] The results of a comparative analysis of all of the forms of HRP by the RI method using KI and ABTS as substrates are given in Table 1, and the change in the substrate specificity of the mutant forms during RI are presented in Fig. 9.

The data obtained show that replacement of the hydrophobic Phe residue by the His residue results in an increase in the conformational flexibility of the active center, which results in broadening of the range of stability of the "molten globule" state (D_{plat} attains

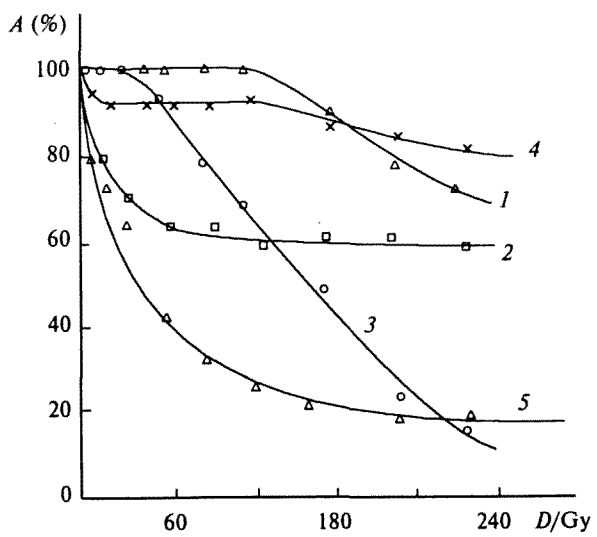

Fig. 9. Radiation-induced inactivation of F143E (*1—3*)[23] and F41H (*4*, *5*),[19] Concentration 10^{-7} mol L^{-1}, pH 8.0, Tris-HCl buffer. Substrates: KI (*1* and *4*), ABTS (*2* and *5*), *o*-phenylenediamine (*3*).

Table 1. Comparison of radiation-chemical parameters (D/Gy) of mutant forms of HRP (10^{-7} mol L^{-1}, Tris-HCl buffer, pH 8.0)

Form of horse-radish peroxidase	Substrate	D_{ind}	D_{80}	D_{50}	D_{37}	D_{plat}	Level of residual enzyme activity on plateau (%)
NHRP	KI	30	105	200	225	—	—
	ABTS	20	45	125	210	—	—
RHRP	KI	—	60	285	~550	5—30	90
	ABTS	—	<1	30	75	8—20	55
F41H	KI	—	300	~550	—*	1—120	95
	ABTS	—	2	45	80	—	—
F143E	KI	120	240	360	—*	—	—
	ABTS	30	90	165	205	—	—

* The value was not attained.

120 Gy). However, in F41H, this state differs in structure from the "molten globule", because the state stable with respect to ABTS was not observed. This is evidently associated with the higher value of S_{acc} of the active center of the mutant on the whole, and, hence, higher "availability" of the ABTS binding site.

Apparently, introduction of a negatively charged Glu residue (F143E) prevents formation of the "molten globule" state (see Table 1). However, the possibility of a dose shift in the range of its appearance exists, because this shift to lower doses was observed with a decrease in the concentration of RHRP.[17] According to the kinetic data[36,37] and our investigations,[29] Phe143 creates a hydrophobic barrier at the entrance to the active center, and, hence, the appearance of the Glu residue at this site should impede an attack by the negatively charged particles formed outside of the active center (which occurs during radiolysis) of the heme on the whole. The values of the radiation-chemical parameters in the case of ABTS as the substrate (see Table 1) show that the attack on the protein part is particularly impeded. Therefore, one can assume that the negatively charged products of radiolysis of water, *viz.*, e^-_{aq} and/or $O_2^{-\cdot}$, are responsible for formation of the "molten globule". Of these two products, e^-_{aq} should be preferred, because the superoxide radical is also generated on inactivation of HRP by H_2O_2, however, as can be seen from Fig. 10,[22] no stable intermediate of the "molten globule" type is formed on the action of H_2O_2.

Although introduction of the Glu residue does not result in the formation of the radiation-stable, intermediate state of F143E on RI, the overall radiation stability of this form of HRP, expressed by D_{ind} and D', is quite high. For ABTS, this value is close to that for RHRP (with account of an intermediate, *i.e.*, the value of $D' = D_{ind} + D_{plat}$ is considered) and it is substantially higher than that for F41H, where the accessibility of the active center increases by *ca.* four times (as follows from the changes in the radiation-chemical parameters), which substantially increases the damage of the ABTS binding site. It is interesting that with respect to the iodide anion, the range of stability of mutant forms (D' for F41H) is almost identical and *ca.* four times broader than those for NHRP and RHRP. This additionally confirms that the iodide anion reacts directly with the porphyrin ring of heme and also demonstrates the absence of any significant effect of $O_2^{-\cdot}$. The latter is due to either a low radiation-chemical yield of its formation under given conditions or a particularly high rate of its reaction outside of the active center (for example, with Phe142).

The comparison of radiation-induced inactivation of HRP and inactivation by H_2O_2 elucidates the role of the

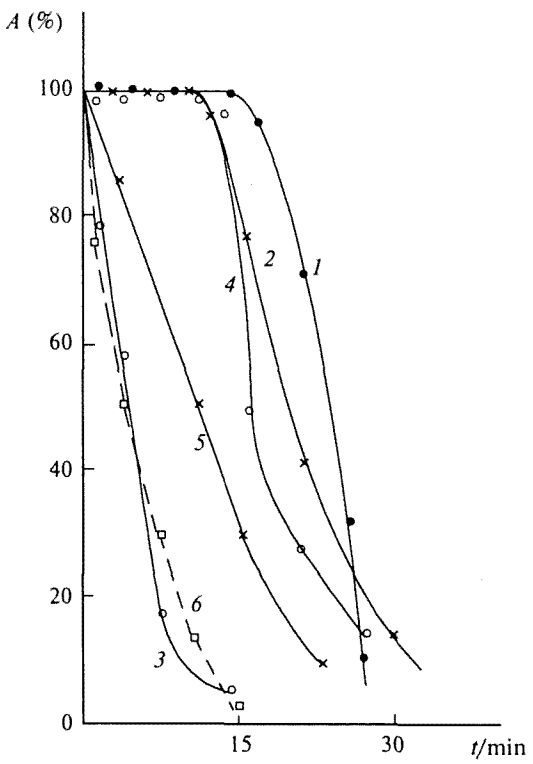

Fig. 10. H_2O_2-induced inactivation of NHRP (*1—3*) and RHRP (*4—6*). Concentration of 10^{-7} mol L^{-1}, 0.01 *M* Tris-HCl buffer, pH 8.0, $5 \cdot 10^{-3}$ *M* H_2O_2.[22] Substrates: KI (*1* and *4*), ABTS (*2* and *5*), *o*-phenylenediamine (*3* and *6*).

Table 2. Comparison of parameters of inactivation of mutant forms of HRP by H_2O_2 (5 mol L^{-1}, Tris-HCl, pH 8.0)

Form of horseradish peroxidase	Substrate	t_{ind}	t_{50}	t^*
		min		
NHRP	KI	12	25	30
	ABTS	6	19	35
RHRP	KI	10	16	30
	ABTS	—	10	25
F41H	KI	—	4	10
	ABTS	1	8	10
F143E	KI	—	6	12
	ABTS	4	11	15

* Time of complete inactivation.

superoxide radical, $O_2^{-\cdot}$, which can be formed in both cases, but in different yields. Note, that H_2O_2 can generate $O_2^{-\cdot}$ directly in the active center of the enzyme, unlike the radiation-induced product, which appears outside of it and duffuses into the active center. The comparison of the action of H_2O_2 (Table 2, Fig. 10) and the radiation effect (see Table 1) on different mutant forms of HRP reveals some important aspects. First, the stabilities of the iodide-binding site with respect to H_2O_2 (D_{ind}) are similar for NHRP and RHRP and drop strikingly for the mutant forms, which is opposite to the radiation effect. In general, HRP can undergo the following changes in inactivation:

$$HRP^{3+} + H_2O_2 \longrightarrow HRP^{4+}\text{—}O\,, \quad \mathbf{1}$$

$$HRP^{3+} + H_2O_2 \longrightarrow HRP^{3+}\text{—}OH\,, \quad \mathbf{2}$$

$$HRP^{3+} + H_2O_2 \longrightarrow HRP^{2+}\text{—}OOH \quad \mathbf{3}$$

to form compounds **1**—**3**, which can be revealed by the differences in their UV spectra.

It was previously[22] assumed that H_2O_2 and radiation-induced inactivations of HRP yield compound **1**, and the mechanisms of these processes are similar. However, more detailed spectral studies of irradiated HRP demonstrated a major difference between them. It is the formation of compound **2** under the action of radiation and of compound **1** in the presence of H_2O_2.[40] As can be seen from Fig. 11, *a*, an absorption maximum at λ = 418 nm and a small increase in absorption in the range of 520—560 nm appear instead of the peak at λ = 403 nm characteristic of NHRP. This is believed to point to the appearance of compound **2**, which is formed rather rapidly, and at doses above 100 Gy (see Fig. 11, *b*), the ratio between NHRP and irradiated HRP is practically constant. It should also be noted that

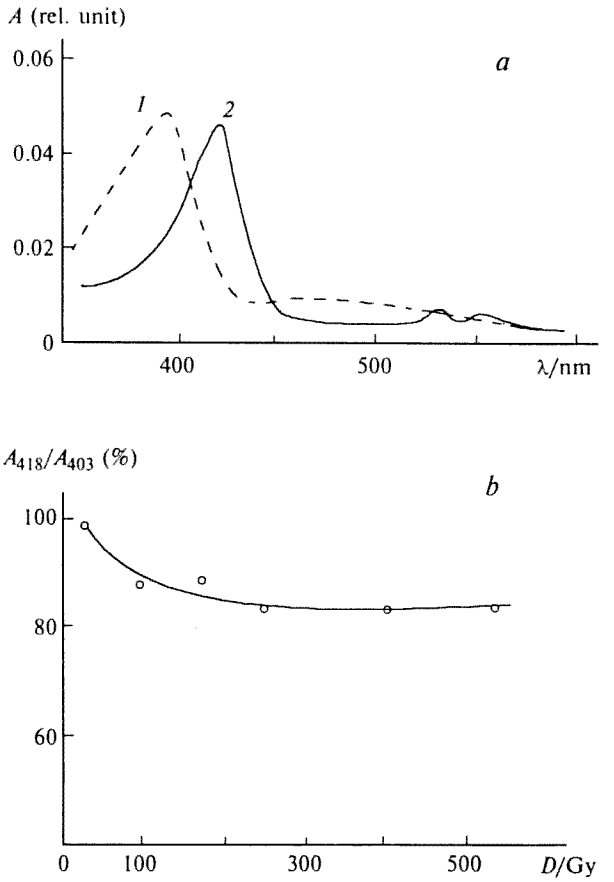

Fig. 11. Spectral changes observed in radiation-induced inactivation of a 10^{-6} *M* solution of HRP in Tris-HCl buffer (pH 8.0). *a*. 10^{-6} *M* solution of NHRP (*1*) and compound **2** (*2*) after irradiation with a dose of *ca.* 30 Gy. *b*. Changes in the ratio of absorptions (A_{418}/A_{403}) during radiation inactivation.

we did not observe the formation of compound P-670 under conditions of RI,[22] although it was described as being formed through one of the pathways of the inactivating action of H_2O_2.[39] Thus, one can assume that the superoxide radical generated inside the active center mainly reacts with the porphyrin ring and any increase in the accessible surface of the active center (in different forms of HRP) increases the efficiency of the inactivating effect of H_2O_2, particularly with respect to the iodide anion; here, compound **1** is formed. At the same time, radiation more strongly affects the protein component (*i.e.*, breaks binding of hemin with the protein) than heme itself (because the active products exist outside the active center). This was additionally confirmed by the method of second derivatives of UV spectra[19] (Fig. 12), because the intensity of the peaks in the second derivative of the UV spectrum decreases more rapidly for the protein moiety. In this case, the formation of compound **2** is apparently favored.

It should be assumed that the superoxide radical does not play the critical role in conformational transformations of HRP and that this role is played by $^{\cdot}OH$

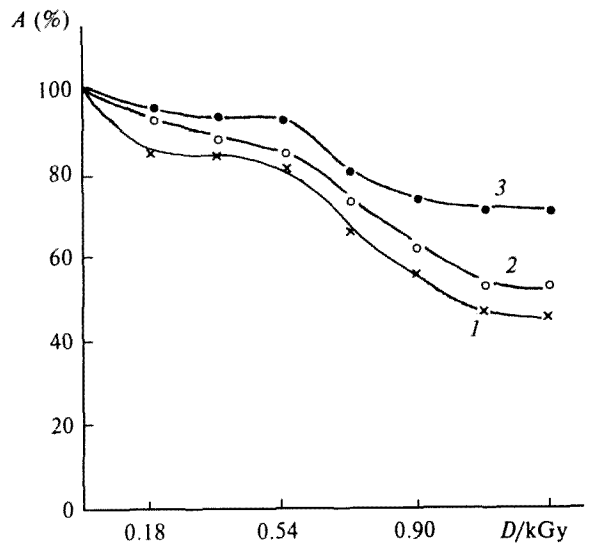

Fig. 12. Changes in the enzymic activity of (*1*) NHRP ($2.5 \cdot 10^{-5}$ mol L^{-1}, phosphate buffer, pH 6.0) and in intensity of peaks of the second derivatives of UV spectra in the protein (*2*) and heme (*3*) moieties.[22]

radicals (attack on a heme-protein complex) and, in some cases, by e^-_{aq} (attack on the porphyrin ring). This conclusion is in agreement with the simplest calculations. It is known[27] that the ratio of radiation-chemical yields of the formation of reactive species in radiolysis of water is as follows: $G_{OH} : G_{e^-_{aq}} : G_{H_2O_2} \approx 2.8 : 2.8 : 0.7$. For $D = 30$ Gy ($t_{irr} = 10$ min), according to the highest estimations, the concentration of H_2O_2 cannot exceed 10^{-7} mol L^{-1}. However, judging from the observed radiation-induced inactivation, insignificant amounts of H_2O_2 and superoxide radicals, which are intermediate products of radiolysis, can completely transform HRP to compound **2**, if its concentration is 10^{-6} mol L^{-1}. This shows the participation of other radiation-induced species in generation of compound **2**. Taking into account the published data,[40] this is most probably $^{\cdot}OH$ radicals. At a concentration of NHRP equal to 10^{-6} mol L^{-1}, the enzyme transforms completely into compound **2**; when its concentration is increased to $2.5 \cdot 10^{-5}$ mol L^{-1}, a mixture of NHRP and compound **2** is observed. It should be noted that compound **2** is dynamically stable in the dose range of *ca.* 90—500 Gy, although in the postradiation period, its partial, reversible transition into the initial but inactive form of NHRP was observed,[22] whereas the changes caused by H_2O_2 are irreversible. It is interesting to note that for tobacco peroxidase having a Glu residue instead of a Phe residue at the entrance into the active center, interaction of the enzyme with an equimolar amount of H_2O_2 gives compound **2**, and in an excess of H_2O_2, a fast **1** → **2** → **3** transition occurs so that compound **2** could virtually not detected.[41] This confirms the influence of the Glu residue and, hence, the negative charge on the heme—protein conformation.

Thus, on the basis of the above data, the following conclusions can be made, which gives grounds for the use of the RI method for studies of the structure and properties of enzymes.

1. In the pH range of 7.0—7.5, conformational transitions are observed as the accessible surface area of the active center increases; they are perhaps connected with deprotonation of His residues.

2. The presence of a large number of Trp and Tyr residues, some of which are not involved in regulation of conformational changes of the active center, and also of carbohydrate fragments favors the autoprotective mechanism, which can lead to formation of unsmooth dose dependences. The higher efficiency of this mechanism, the higher the rate of subsequent inactivation, *i.e.*, an increase in G_{in} is observed (the effect of accumulation of damages).

3. An increase in the conformational flexibility of the active center increases the degree of the reversible effect of radiation, which results in an increase in the stability of the molecule and regions of activation can appear (an increase in the efficiency of catalysis with respect to some substrates).

4. The change in substrate specificity under the effect of radiation is relared directly to the accessibility of the substrate-binding sites, and its study provides direct information on the structure of the molecule and its catalytic properties.

5. When low doses of radiation are used, a "conformational imbalance" in the molecule is observed, which in some cases can result in alternation of the ranges of activation, inactivation, and stability on the dose curves.

6. There are some reasons to assume that if only a few (or even one) Trp and Tyr residues are present in the amino acid sequence of the enzymes, they are responsible for the conformational flexibility of the active center and are usually involved in its immediate environment.

7. The effect of radiation on heme-containing enzymes results in preferred attack of $^{\cdot}OH$ radicals on the heme—protein structure and of e^-_{aq} on the porphyrin ring. The difference in the sites of formation of the reactive species (inside the active center (H_2O_2) or outside it (radiation)) affects the irreversibility (or reversibility, respectively) of their interaction with the enzyme and increases the rate of inactivation in the former case. The presence or absence of charged amino acid residues at the entrance to the active center becomes of great importance.

8. The most precise information on the structure of enzymes in solution can be obtained by combining the methods of molecular engineering and RI.

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