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Enzyme Activation and Inactivation Induced by Low Doses of Irradiation

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Abstract

Activation phenomenon has been observed with two sets of enzymes under the conditions of low dosage irradiation. Activation was registered for angiotensin-converting enzyme under in vitro γ-irradiation (0.662 MeV, pulse duration approx 10 s) at dose levels of 1–3 Gy and under X-ray irradiation (approx 9 keV, pulse duration approx 10^{-9} s) at dose levels of 2×10^{-5} Gy. An activation effect has also occurred for native and recombinant horseradish peroxidase and tobacco peroxidase under γ-irradiation. The phenomenon observed is rationalized in terms of a kinetic model suggesting the existence of at least one activated enzyme conformation induced by radiolysis. The activity oscillations registered in dense plasma focus experiments were rationalized using the same model with the corresponding kinetic equation converted into the form describing the decaying oscillations caused by exciting force. The model analysis is presented.

Index Entries: Radiation activation; angiotensin-converting enzyme; peroxidase; dense plasma focus; γ-irradiation; kinetic model; activity oscillation; periodic process.

Introduction

Radical processes play a crucial role in the development of medical pathologies and, in particular, cancer *(1)*. It is known that radiation-induced cancer simulates the mechanisms of spontaneous and chemically induced cancers *(2)*. The most studied are the processes of radical damage

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of DNA. However, it is clear that radical damage of enzymes and enzyme systems should also negatively affect the organism. This laboratory developed a new scientific procedure that can be called radioenzymology *(3)*. The goal of studies performed with this new procedure is experimental and theoretical modeling of radical damage of enzymes for predicting its initiation, development, and prevention.

Radioenzymology operates with enzyme water solutions. Their irradiation yields the active particles of water radiolysis whose spectrum depends on the irradiation conditions. The dose power and the character of dose accumulation are important parameters of irradiation. In other words, the same dose can be reached either with a relatively small-dose power and a comparatively long irradiation period or with a high-dose power in a short time. That is why different types of radiation sources may expand our knowledge about the enzyme dose response and, therefore, about the enzyme properties. Radioenzymological studies have been shown to be very sensitive to minor changes in the enzyme structure: in the case of horseradish peroxidase the investigation of radiation-induced inactivation allowed us to detect changes in the enzyme folding *(4)*.

Enzyme and enzyme systems exhibit a property to be activated under some conditions (chemical agents and UV). This property is of general importance for their functioning in vivo. We have shown for the first time the activation effect of ionizing radiation on angiotensin-converting enzyme (ACE) *(5)*, which is a key enzyme in the system of blood pressure regulation and presents a metalloglycoprotein of hydrolase class (EC 3.4.15.1).

The present study focuses on radiation-induced activation of ACE and its comparison with the dose response of heme-containing plant peroxidases (EC 1.11.1.7) under the in vitro irradiation conditions with arbitrary and really small doses generated by γ- and X-ray sources. A mathematical model developed in this study allowed us to demonstrate that activation is a general feature of enzyme dose response.

Materials and Methods

Enzymes and Enzyme Assays

ACE

Electrophoretically homogeneous ACE (MW 180 kDa) from bovine lungs was isolated and purified as in ref. *6*. The enzyme contained about 98% of active molecules as determined by stoichiometric titration *(7)* with a specific competitive inhibitor, lisinopril (Sigma). ACE enzymatic activity was determined with 10^{-5} *M* carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine (Cbz-Phe-His-Leu) (Serva, Germany) as a substrate in 0.05 *M* phosphate buffer, pH 7.5, containing 0.15 *M* NaCl, 25°C, using *o*-phthalaldehyde modification of His-Leu as a reaction product *(8)*.

Peroxidases

Native horseradish peroxidase C (HRP, MW 44 kDa) was purchased from Biozyme, and recombinant wild-type HRP (MW 34 kDa) was produced from *Escherichia coli* inclusion bodies by the method developed by us earlier *(9)*. Tobacco anionic peroxidase (TOP, MW 36 kDa) from *Nicotiana sylvestris* transgenic plants overexpressing the enzyme was purified as described in ref. *10*. The measurements of peroxidase activity were perfomed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and guaiacol (Sigma) as substrates using a Shimadzu UV 120-02 spectrophotometer (Japan) at 25°C as follows:

1. 0.05 mL of ABTS solution (8 mg/mL) and an aliquot of the enzyme were added to 2 mL of 0.1 *M* Na-acetate buffer (pH 5.0), the reaction was initiated by the addition of 0.1 mL of hydrogen peroxide (100 m*M*). A molar absorptivity of ABTS oxidation product was taken equal to 36800 L/mol/cm at 405 nm *(10)*.
2. 0.15 mL of guaiacol water solution (1 mg/mL) and an aliquot of the enzyme were added to 0.1 *M* Na-acetate buffer (pH 5.0, 0.2 mL), and the reaction was initiated by the addition of 0.1 mL of hydrogen peroxide (100 m*M*). A molar absorptivity of guaiacol oxidation product was taken equal to 25,500 L/mol/cm at 436 nm *(11)*.

Irradiation

Two irradiation sources were used throughout the experiments: a standard γ-source (^{137}Cs, 0.662 MeV, P_g = 0.05 Gy/s) and a miniature dense plasma focus source (DPF) with an energy storage of 200 J. All DPF sources provide a powerful pulse X-ray irradiation with a broad spectrum and with the intensity depending on the energy storage. DPF X-ray spectrum exhibits some special characteristics as compared to X-ray tubes. X-irradiation generated by DPF can be separated into two components, a hard one resembling X-tubes and an intense soft component originating from the hot pinched plasma (Fig. 1). DPF X-irradiation of enzyme solutions was performed using copper foil (90 μm) and aluminum plate (1-mm) filters whose transparency curves are presented in Fig. 2.

The soft component present in the input DPF spectrum disappears after the passage through the aluminum filter (Fig. 3), or in other words, Al is completely opaque to this component. So, the principal peculiarity of copper foil filter is that there is a small "notch" in the transmission curve in the region of 8–9 keV (Fig. 3), the so-called K-edge transparent to 8 keV photons. Nothing like that occurs with aluminum filter (Fig. 3) and so, the copper K-edge irradiation is almost monochromatic. This allows us to study the influence of quasi-monochromatic X-ray radiation with photon energies of 8.98 keV (λ = 1.381 Å) on enzymes.

The irradiation was performed at 18–20° C. The absorbed dose was determined with thermoluminescent detectors based on LiF activated with Mg, Cu, and P according to the protocol (*12*). Dosimeters were placed into

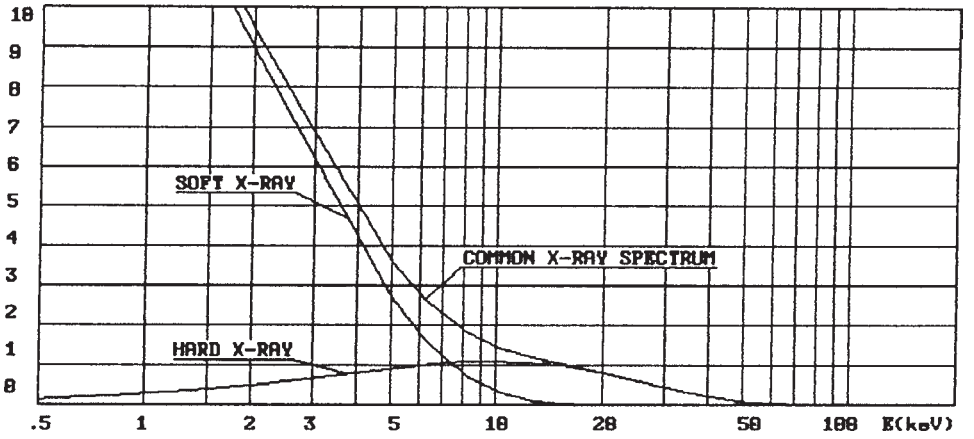
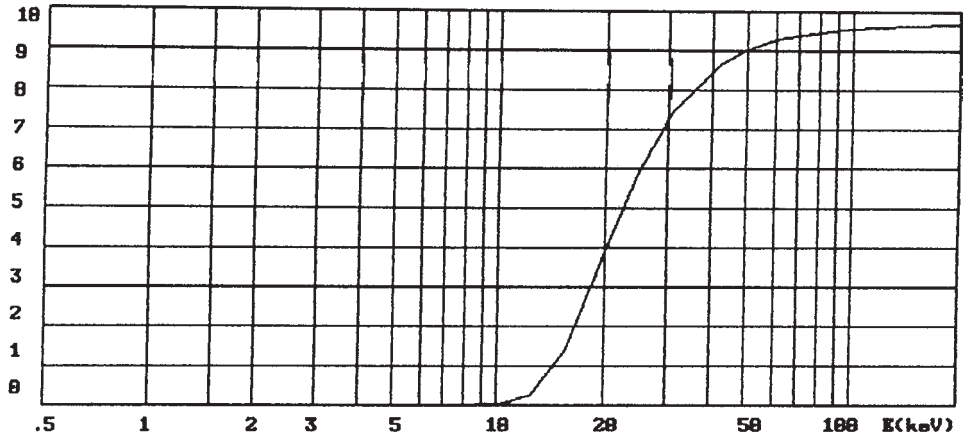
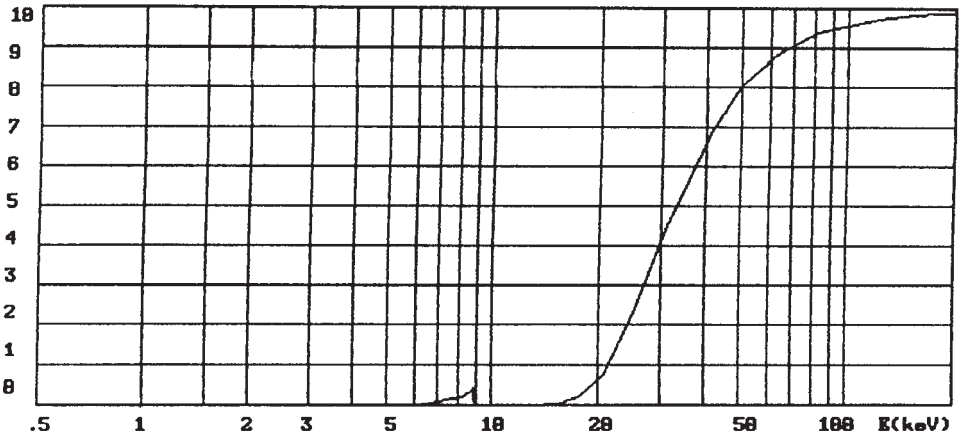


Fig. 1. Typical X-irradiation spectrum of DPF. Number of photons in arbitrary units is shown in ordinate.



A



B

Fig. 2. The transparency curves for (A) aluminum 1-mm thick and (B) copper 90-μm thick filters.

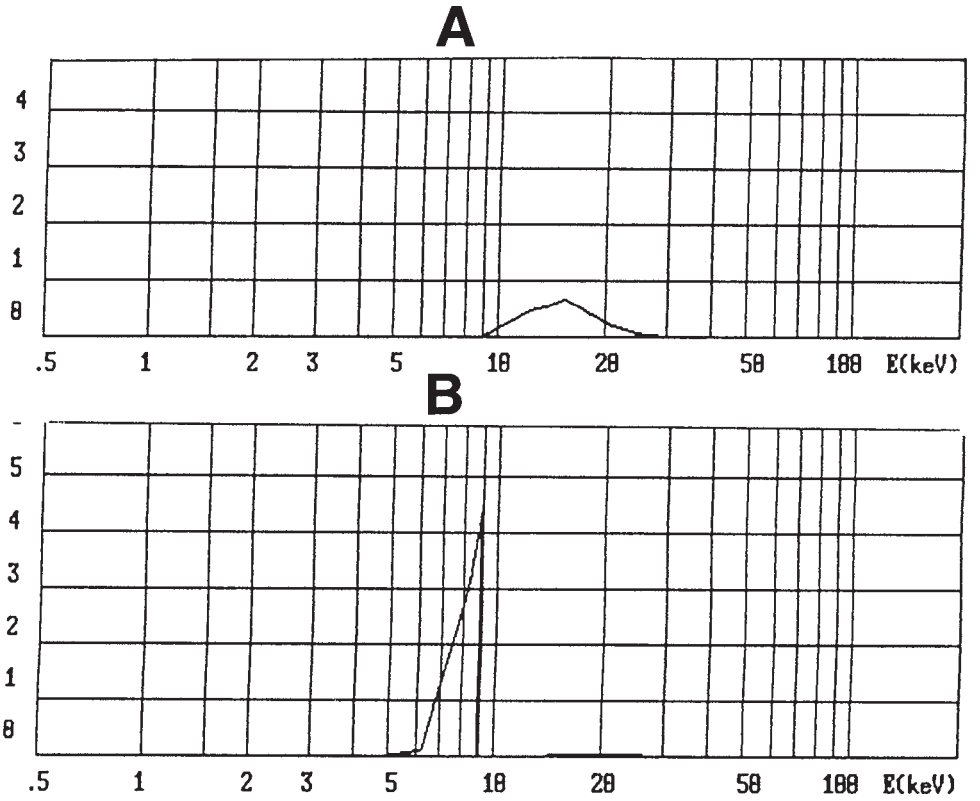


Fig. 3. The spectrum DPF X-irradiation passed through (A) Al-filter, polyethylene 0.66-mm thick and 10 mm water layer, (B) Cu-filter, polyethylene 0.665-mm thick and 10-mm water layer.

a polyethylene cover 1 g/cm thick. The measurements were performed using a thermoluminescent analyzer Harshaw TLD system-4000.

Second-Derivative Spectrophotometry

Damage of aromatic amino acid residues was determined quantitatively by following the changes in the peak intensities in the second derivative of the spectra of the proteins at λ = 290.5 nm for tryptophan and λ = 284.2 nm for tyrosine *(13)*. The spectra of ACE (0.15 mg/mL) and corresponding second derivatives were obtained with a Shimadzu 265 FW spectrophotometer.

Results and Discussion

Activation Induced by γ-Source Irradiation

ACE

Typical radiation-induced activation in the case of ACE is illustrated by the data presented in Fig. 4. The dose corresponding to the activation

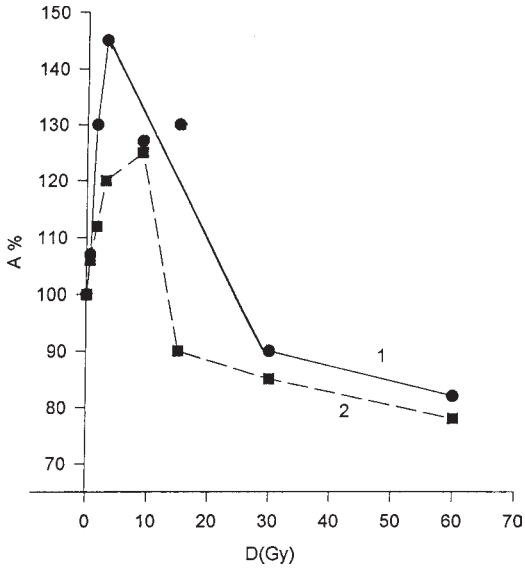


Fig. 4. Changes in catalytic activity of γ-irradiated 10^{-8} *M* ACE solution at pH 6.0 (1) and 7.0 (2), phosphate-borate buffer, 0.15 *M* NaCl.

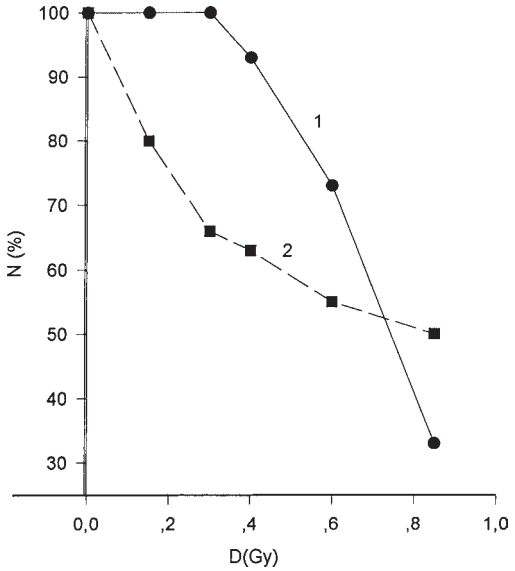


Fig. 5. γ-Irradiation of 10^{-6} *M* ACE solution, phosphate-borate buffer, 0.15 *M* NaCl, pH 7.5: 1, activity; 2, Trp and Tyr content estimated by second derivative of UV-spectra.

effect (3 Gy) can be considered as arbitrary small in the case of irradiation in vitro. The changes in the irradiation conditions (pH, enzyme concentration, solution components) can either shift the activation peak or lead to its disappearance. The effect of pH on the activation peak in the case of ACE is demonstrated in Fig. 4 (curves 1 and 2).

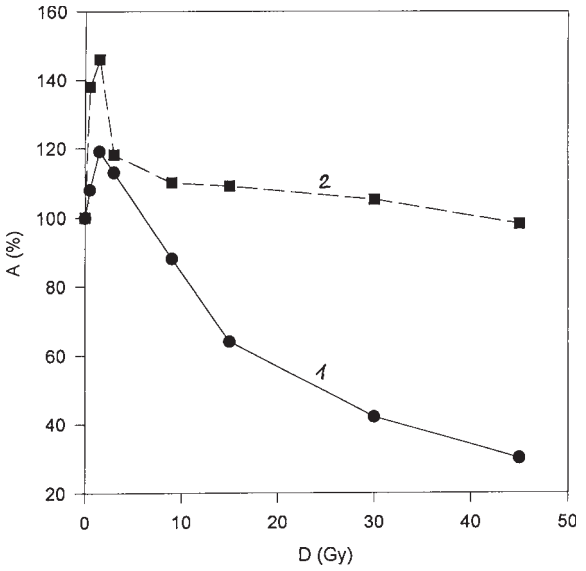


Fig. 6. γ-Irradiation induced changes in horseradish peroxidase catalytic activity. 1, native HRP; 2, recombinant HRP pretreated with 10^{-6} *M* H_2O_2. 10^{-7} *M* enzyme solution in acetate buffer, pH 6.0, substrate guaiacol.

The increase in the enzyme concentration results in principal changes in the character of the dose response curve. For instance, we have shown earlier *(5)* that the activation peak could be observed after the initial loss of the enzyme activity (the so-called "secondary" activation). Quite often, the dose response curves had a plateau, lag-period, substituting for the activation (Fig. 5, curve 1). It is interesting that there are no detectable activity changes in the course of the lag-period, whereas the enzyme modification takes place according to the spectral studies. Conformational changes in many enzymes (proteinase in particular) are known to be caused by modification/destruction of Trp and Tyr residues *(14)*. Second-derivative UV-spectrophotometry permits following the modification/destruction of these aromatic amino acid residues *(13,15)*. Figure 5, curve 2, presents the data calculated from the second-derivative UV-spectrum of the irradiated ACE. It is unequivocally shown that approx 35–40% of Trp and Tyr residues in ACE are lost within the lag-period corresponding to the former catalytically stable form.

HRP

The dose response curve of native HRP also exhibits 20% activation (D_{max} = 1.5 Gy) with guaiacol as a substrate (Fig. 6, curve 1). The recombinant deglycosylated HRP refolded from inclusion bodies showed no activation (data not shown). However, it demonstrated a 45% increase in activity toward guaiacol at the same dose (D_{max} = 1.5 Gy) being preliminary treated with equimolar hydrogen peroxide (Fig. 6, curve 2). The treatment with hydrogen peroxide yields the oxidized active center, which is subse-

quently reduced by the protein itself. The data obtained demonstrate the changes in the active center accessibility induced by glycosylation and pretreatment with hydrogen peroxide. The earlier work on the HRP-catalyzed oxidation of iodide rationalized the reaction mechanism in terms of an equilibrium between two HRP conformations, the so-called "open" and "closed" *(16)*, to account for the different accesibility of the active center. Our data also indicate the probable existence of two conformers of recombinant HRP with different sensitivity to irradiation. We did not observe radiation-induced activation of HRP using other substrates, although in some conditions the dose response curves had lag-period (like that found for ACE) with iodide and ABTS used as substrates *(17)*.

TOP

In the case of anionic TOP the active center is screened by the negatively charged Glu-141 *(18)*, which could be a reason for a significant activation (D_{max} = 45 Gy) observed using guaiacol as a substrate (not shown). The activation phenomenon is stronger in the presence of calcium cations (Fig. 7), which are known to cause the conformational changes in the active center structure *(19)*.

Activation Induced by DPF-Source

The appearance of subtle effects under in vitro irradiation with arbitrary small doses inspired us to decrease the irradiation dose until it was very low and to study the activation phenomenon under conditions corresponding to the constant concentration of radicals in a radiochemical reaction. The latter was achieved using a DPF source with an extremely high power dose. A dose was accumulated within a single pulse (10^{-9} s) or a number of pulses.

The data obtained with DPF irradiation (E_{max} ~8.9 keV, Cu-filter) of ACE and HRP are presented in Fig. 8. DPF irradiation with Cu filter is almost monochromatic (*see* Materials and Methods). In the case of ACE at the dose of 2×10^{-5} Gy, a significant (up to 50%) activation took place at both pH 7.5 and 6.0, which correspond to different conformations of the enzyme and its active center(s) as we discussed earlier *(5)*.

On the other hand, HRP inactivation (with ABTS as substrate) was observed over the same dose interval (Fig. 8). We should emphasize that the increase of an accumulated dose achieved by either a higher dose of a single pulse or a number of pulses did not yield any activation.

The activation effect was not found for both enzymes (ACE and HRP) in the case of Al-filter or with X-ray tubes (data not shown). These observations indicate the dependence of the activation phenomenon on the photon energy and, consequently, on the density of the radicals in track. It is difficult to determine the absorbed dose values since soft X-irradiation is absorbed by both solution and dosimeter. Therefore, the doses of soft X-irradiation are approximate, which is in contrast to hard X- and γ-irradiation whose doses can be determined with high accuracy. The observed

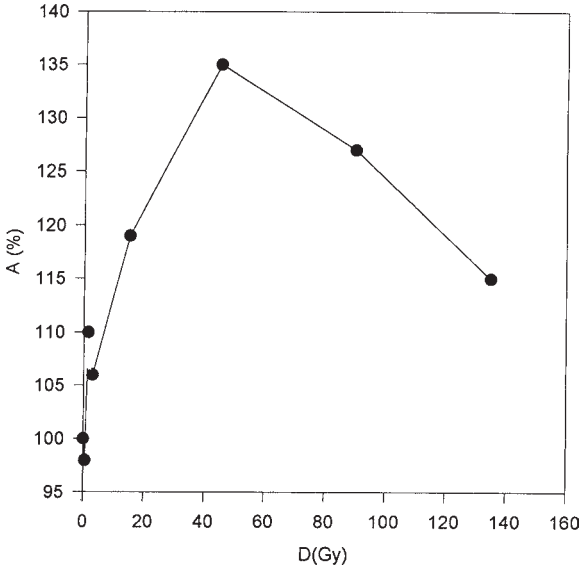


Fig. 7. γ-Irradiation induced changes in tobacco peroxidase activity. 10^{-7} *M* enzyme solution in acetate buffer, pH 6.0, in the presence of 5×10^{-2} *M* $CaCl_2$, substrate- quaiacol.

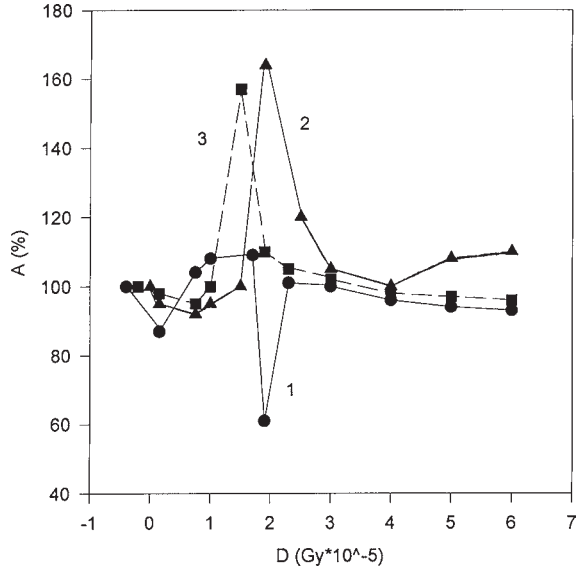


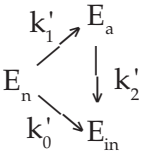
Fig. 8. DPF X-irradiation induced changes in enzymatic activity (Cu-filter, single pulse): 1, native HRP, 10^{-7} *M*, phosphate buffer, pH 6.0, substrate ABTS; 2, ACE, 10^{-8} *M*, phosphate-borate buffer, pH 6.5, substrate Cbz-Phe-His-Leu; 3, ACE, 10^{-8} *M*, phosphate-borate buffer, pH 7.5, substrate Cbz-Phe-His-Leu.

oscillations of the enzymatic activity (Fig. 8) point to the periodicity of the activation/inactivation phenomenon in the course of radiolysis. What process is predominant depends on the specific enzyme and irradiation con-

ditions, and also on the substrate used because the induced conformational changes affect specific substrate-binding centers. Oscillations of enzyme activity are remarkable and reproducible and they are photon energy-dependent. Therefore, the data obtained with DPF allow us to conclude that the dose response curves for the studied enzymes have special points corresponding to the reversible imbalance (activation–inactivation) of the enzyme molecule.

Mathematical Model of Activation

Thus, the enzyme activation phenomenon can be considered as rather common. To describe the radiation-induced changes in the enzyme activity, we can propose the existence of an activated conformation in accordance with the following scheme:



where E_n, E_a, and E_{in} are native, activated, and inactivated enzyme forms, respectively; k'_0, k'_1, k'_2 are the rate constants of the corresponding radical-induced steps in Scheme 1.

Let us propose that the apparent specific activity of native and activated enzyme forms are $V_n = 1$ and $V_a > 1$, whereas the inactivated enzyme lacks the catalytic activity ($V_{in} = 0$).

The system of differential equations corresponding to the simplified process presented in Scheme 1 is

$$dE_n/dt = -(k_1' + k_0')\, E_n\, [R] \tag{1A}$$

$$dE_a/dt = (-k_2' E_a + k_1' E_n)\, [R] \tag{1B}$$

under the initial conditions $E_n = 1$ and $E_a = 0$ ($t = 0$), [R] is the concentration of active products of radiolysis. The further simplification assumes the constant concentration of radicals in the course of radiolysis. This assumption is valid only for high dose power and minimum irradiation time. These conditions are realized with DPF source and in the beginning of γ-irradiation in the absence of radical acceptors. The solution of the system 1 expressed for

$$V_E = E_n + V_a E_a \tag{2}$$

(corresponds to the measured enzyme activity) is presented below, where K_1, K_2, and K_0 substitute for $k_1'[R]$, $k_2'[R]$, and $k_0'[R]$, respectively:

$$V_E = [V_a K_1/(K_0 + K_1 - K_2)] \exp(-K_2 t) + [1 - V_a K_1/(K_0 + K_1 - K_2)] \exp[-(K_0\ K_1)t] \tag{3}$$

It is logical to assume that the inactivation rate constants for both native and activated enzymes are close to each other, or in other words that

$K_0 \sim K_2$ and $K_2 - K_0 << K_1$. Then the expression for the activity changes in the beginning of irradiation ($t = 0$) will be as follows:

$$dV_E/dt_0 = -V_a K_2 + (V_a - 1)(K_0 + K_1) \quad (4)$$

If the activation is much less probable than inactivation ($K_1 << K_0$), the criterion for the activation will be $K_1/K_0 > 1/V_a$ and $V_a >> 1$, or, in other words, we must propose the existence of superactive conformations. The existence of enzyme superactive conformations is principally possible; in particular, they were shown in micellar enzymology *(20)*. However, if the probability of activating and inactivating radical modifications induced by radiolysis is comparable ($K_1 < K_0$ and $V_a > 1$), there is no need to suppose the existence of superactive conformations to rationalize the observed activation effects.

Oscillations of enzymatic activity observed for ACE and peroxidases may indicate the periodicity of the activation/inactivation processes. We can rationalize this observation using the above model if Eq. 1 can be converted to an equation corresponding to decaying oscillations caused by an exiting force. This type of an equation will be a general one including all possible variants of enzyme dose response. This is, indeed, possible if we differentiate both equations of Eq. 1, multiply the second equation by V_a, and add them taking into account Eq. 2:

$$(d^2V_E/dt^2) + K_2 V_a (dE_a/dt) + [(K_0 - K_1)(V_a - 1)](dE_n/dt) = 0 \quad (5)$$

Adding and substracting Eq. 1B multiplied by $[V_a (K_0 + K_1 - K_1 V_a)]$ from Eq. 5, we obtain after some manipulations Eq. 6, which presents a linear nonhomogeneous equation with constant coefficients:

$$(d^2V_E/dt^2) + [K_0 + K_1(1 - V_a)](dV_E/dt) + K_2 [K_0 + K_1(1 - V_a) - K_2)] V_E = (V_a K_1 + K_2)\{[K_0 + K_1 (1 - V_a)] - K_2\} \exp[-(K_0 + K_1) t] \text{ at } E_o = 1 \quad (6)$$

The solution of this type of equation is described in ref. *21*. We can introduce a time transformation of $t \rightarrow \rho t$ type, where ρ corresponds to the changes in the dose power. This will not change the character of the dose response curve but it broadens or compresses it depending on the ρ value (assuming all other coefficients independent of dose power). Or, in other words, the activation/inactivation peak can change the position and the amplitude. Thus, Eq. 1 leads to Eq. 6 presenting the decaying oscillations caused by an exiting force.

Introducing

$$\alpha = \frac{[K_0 + K_1(1 - V_a)]}{2}$$

and

$$w_0^2 = [K_0 + K_1(1 - V_a) - K_2] K_2$$

Eq. 6 can be rewritten as follows:

$$V_E'' + 2\alpha V_E' + w_0^2 V_E = 0 \quad (7)$$

Let us look for the solution of Eq. 7 in the form $V_E = e^{-wt}$. In this case Eq. 7 transforms to Eq. 8:

$$w^2 + 2\alpha w + w_0^2 = 0 \quad (8)$$

with the solutions $w_{1,2} = -\alpha \pm \sqrt{\alpha^2 - w_0^2}$

The analysis of solutions gives five possibilities:

1. If $\alpha > 0$, $w_0^2 > 0$, and $\alpha^2 > w_0^2$, we have an exponential inactivation as a result of two decaying exponents: $E = A\exp(-w_1 t) + B\exp(-w_2 t)$.
2. If $\alpha > 0$, $w_0^2 > 0$, and $w_0^2 > \alpha^2$, we have an equation describing decaying oscillations.
3. If $\alpha < 0$, $w_0^2 > 0$, and $w_0^2 < \alpha^2$, we have two growing exponents.
4. If $\alpha < 0$, $w_0^2 > 0$, and $w_0^2 > \alpha^2$, we have an equation of growing oscillations.
5. If $w_0^2 < 0$, the equation contains one growing and one decaying exponents resulting in the activity increase.

Cases 3–5 correspond to the activation process that is periodic, in particular, case 4.

Conclusions

Thus, the probability of periodic processes is lower compared to the appearance of a single activation peak. It is clear from our analysis that the appearance and disappearance of activation peaks and the doses at which they can occur depend on irradiation conditions (in particular, on the values of the enzyme reaction rate constants) and the purity of the enzyme. More important is that activity oscillations caused by special irradiation conditions indicate the existence of molecular mechanisms of enzyme response to irradiation. The key feature of this mechanism is periodicity of inactivating and activating radical modifications. We assume that Trp and Tyr residues may play a significant role in these conformational oscillations.

Acknowledgment

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