Chemistry of Natural Compounds and Bioorganic Chemistry

Radiation-induced inactivation of angiotensin-converting enzyme in aqueous solutions

1. The effect of irradiation conditions

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Radiation-induced inactivation of angiotensin-converting enzyme (ACE) was studied. The relationship of this process to damage to aromatic amino acid residues (tyrosine and tryptophan) was demonstrated by the method of second derivatives of UV spectra. In some cases, the dependence of inactivation on dose had an induction period. The comparison of the radiation-induced inactivation parameters of ACE, serine proteinases, and horseradish peroxidase points to a significant stabilizing effect of carbohydrate residues in glycoproteins ("autoprotective mechanism"). At pH 7.5, conformational changes occur in ACE. Low accessibility of the active center of this enzyme was suggested.

Key words: radiation; angiotensin-converting enzyme.

It is known[1] that the method of radiation-induced inactivation makes it possible to obtain information about the behavior of complex biochemical objects in solutions. In particular, it has been widely used for the study of structural features of some proteinases.[2] In the present work the method of radiation-induced inactivation has been used for investigation of specific features of a more complex physiologically important substance, *viz.*, angiotensin-converting enzyme (EC 3.4.15.1), which is a Zn-containing peptidase and a membrane-bound glycoprotein. This enzyme is a key enzyme in the renin—angiotensin—aldosterone and kallikrein—kinin systems, which control blood pressure in an organism. It has been shown that hypertensive states, heart insufficiency, renal pathologies are associated with the level of ACE activity. Information about the role of ACE in disorders of the reproductive function, psychosomatic diseases, *etc.* has appeared.[3] This has attracted special interest to the elucidation of the structural and functional transformations of the enzyme under physical and chemical actions, in particular, under all types of radiation.

The aim of the present work was to study the conditions and particularities of applications of the method of radiation-induced inactivation to ACE.

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Results and Discussion

A previous investigation of serine proteinases (α-chymotrypsin (Ctr) and subtilysine-72 (Sbt)) by the method of radiation-induced inactivation, showed that the $OH^{\cdot}$ radicals produced by radiolysis of H_2O damaged primarily the aromatic amino acid residues, *viz.*, tyrosine and tryptophan, and this process correlated with the loss of enzymatic activity.[4] For Ctr, as a result of structural specificity of the active center, tryptophan residues are of great importance for retention of enzymatic activity, and tyrosine residues are important for Sbt. In some cases, radiolysis initially caused conformational changes in the protein globule, which led to its unfolding, and, consequently, to an increase in the area of the accessible surface, and then to stochastic damage to the accessible amino acid residues in accordance with their radiosensitivity. The radiation-chemical yield of inactivation correlated with the area of the accessible surface of the native enzyme molecule.[5] This initial perturbation of the conformations of proteinases as a result of the destruction of aromatic amino acid residues under the action of radiation has been described not only for serine proteinases, but for other proteinases as well.[2]

The peaks in the second derivatives of the spectra of the proteins (see Experimental) that correspond to the tyrosine and tryptophan residues are well-defined, and the intensities of the peaks are proportional to the content of these amino acids in the molecule.[6] Thus, by following the changes in the intensities of the peaks that correspond to tyrosine and tryptophan, one can follow the kinetics of their destruction during radiolysis.

In Fig. 1 plots of dose-dependent radiation-induced inactivation of ACE and decomposition of tyrosine and tryptophan residues determined from the second derivatives of the spectra are presented. Conditions of irradiation similar to the physiological conditions were chosen: 0.15 *M* NaCl, pH 7.5. It should be noted that decomposition of tyrosine residues and decomposition of tryptophan residues in the ACE molecule apparently proceed at comparable rates. Analysis of the second derivatives of the spectra at the different stages of irradiation indicates that the residues exposed to the solvent (*i.e.*, surface residues) are decomposed first, thus increasing the percentage of hidden residues in the protein globule.

A molecule of ACE contains 35 tyrosine and 32 tryptophan residues.[7] Destruction of *ca.* 30 % of the aromatic amino acids (*i.e.*, 20—22 residues) results in virtually no loss of enzymatic activity, *i.e.*, the catalytically active conformation of the ACE molecule and its active center remain unchanged. Evidently, under these conditions, the tyrosine residue involved in the structure of the ACE active center itself remains undisturbed. This conclusion is in accordance with the previously obtained data that the active center of ACE is buried deep inside the protein globule.[8] Further destruction of the aromatic amino acid residues is accompanied by fast inactivation of the enzyme. Thus, one can assume that there exists a threshold dependence of radiation stability of ACE.

The important feature of the dose dependence of radiation-induced inactivation of ACE is the existence of an induction period, which depends on the enzyme concentration and the pH of the medium (Fig. 2). These induction effects have not been previously ob-

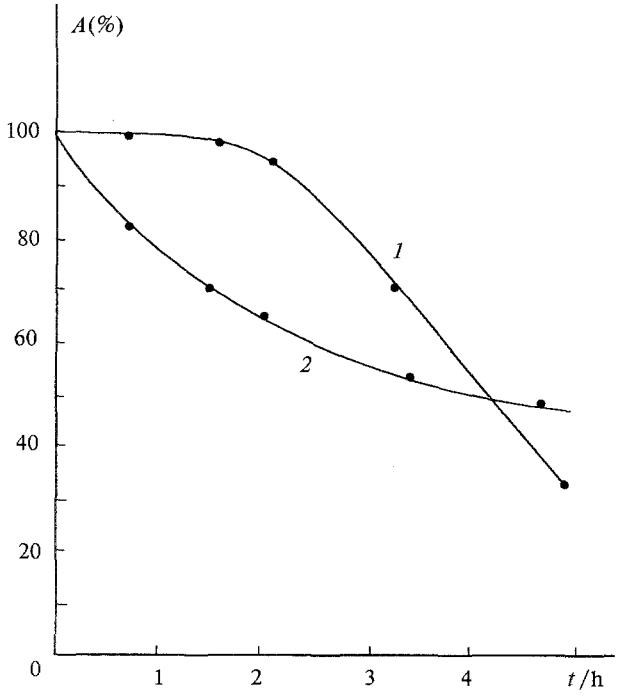

Fig. 1. Changes in enzymatic activity of ACE (*1*) (10^{-6} *M*, 0.15 *M* NaCl, substrate **1**, pH 7.5) and intensities of tyrosine and tryptophan peaks (*2*) in the second derivative of the spectrum of ACE during radiolysis *vs.* time of irradiation (P_γ = 0.05 Gy s^{-1}).

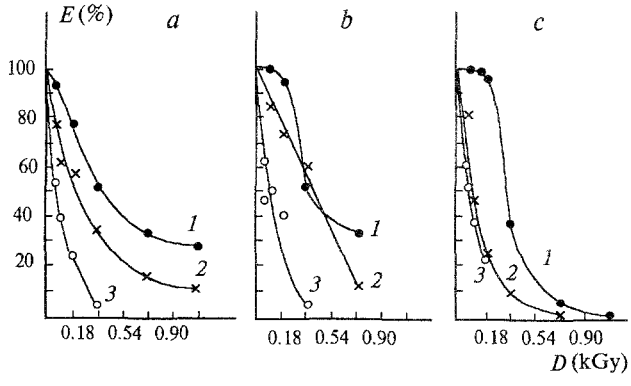

Fig. 2. Concentration functions of radiation-induced inactivation of ACE solutions (0.15 *M* NaCl, substrate **1**) at pH 6.5 (*a*); 7.5 (*b*); 9.0 (*c*): *1*, 10^{-6} *M* (0.15 mg mL^{-1}); *2*, 10^{-7} *M* (0.015 mg mL^{-1}); *3*, 10^{-8} *M* (0.0015 mg mL^{-1}). *E* is the residual activity.

served in the studies of radiation-induced *in vitro* inactivation of other enzymes even at low doses (≤ 3 Gy). In principle, the induction period may be determined by a combination of the following factors:

1. Tryptophan and tyrosine residues can function as protectors in the enzyme molecule. When their number increases in general, *i.e.*, when the initial concentration of the enzyme increases, the number of surface residues, which are insignificant for the conformation of the active center and which can carry out a protective function, also increases. This fact is possibly related to the increase in the dose at which the active center becomes accessible for the attack of the $OH^{\cdot}$ radicals, and to the appearance (and also the extension as the initial enzyme concentration increases) of the induction period. In principle, an analogous process should also proceed in other proteins with the same relative content of aromatic amino acid residues, however, under radiolysis of proteinases no induction period was observed even at small doses (< 3 Gy). It should be noted that the percentages of tyrosine and tryptophan residues in relation to the total number of amino acid residues in Cht and ACE are close to each other and are *ca.* 5 and 7 %, respectively. However, the total number of aromatic amino acid residues in ACE is significantly higher, which results in the strengthening of protection of its active center. Undoubtedly, those tyrosine and tryptophan residues that do not participate in the functioning of the active center are radioprotecting fragments, although it is unlikely that this is the only reason for the appearance of the induction period in the radiolysis of ACE. This conclusion is confirmed by the data obtained by us in the radiolysis of horseradish peroxidase (HRP), which contains only 1.5 % aromatic amino acid residues. In this case, the dose functions had a small induction period (Fig. 3), but this probably is not related to the protective function of the aromatic residues.

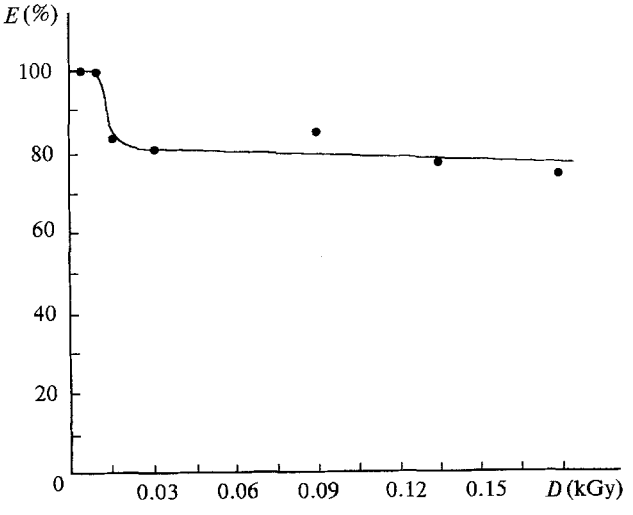

Fig. 3. Dose functions of radiation-induced inactivation of HRP (10^{-6} *M*, phosphate buffer, substrate **3**, pH 7.5). *E* is the residual activity of HP.

2. One significant difference between ACE and the previously studied proteinases is the presence of carbohydrate residues (*ca.* 30 % w/w), which are extremely radiosensitive[9] and are also able to perform the protecting function. It is important that HP, for which, like for ACE, an induction period of the dose dependence of inactivation is observed, is also a glycoprotein (*ca.* 20 % carbohydrate residues).[10]

3. When the enzyme concentration increases, associates can be formed, in which the active centers are protected from the damaging influence of radiation, and a definite number of reactive radicals is consumed for destruction of these associates. The stabilizing role of the association of the enzyme molecules at high concentrations is known for serine proteinases, but the shape of the dose dependence did not change in this case.[11]

Evidently, all three factors enhance the radiation stability of the enzyme, thus creating the autoprotecting mechanism. Elucidation of the exact reasons and conditions for the appearance of the induction period during radiolysis of ACE requires, of course, further studies in the small-dose region. One can assume that the more complex the natural object the higher the degree of its protection as a result of involvement of various mechanisms. Thus, S-shaped dose dependences are widespread for the irradiation of cell populations or organisms *in vivo*.[12]

The radiation damage to a system is conventionally characterized by the dose values D_{80}, D_{50}, and D_{37}, at which 80, 50, and 37 % of the molecules, respectively, remain active. It should be noted that these values are not chosen arbitrarily since they characterize (as has been shown previously for proteinases[5]) definite mechanisms and stages of inactivation. Thus, the initial "conformational" stage is practically completed when 20 % of the molecules are destroyed. When 37 % of the active molecules remain unchanged, it is assumed that only processes destroying the amino acid residues take place, and at the stage of 50 % destruction, the combined mechanism is realized.

Figure 4 shows the dependences of the specified *D* values on the initial concentration of enzymes in acidic and basic media. These data illustrate the threshold character of the inactivation of ACE, because the D_{80} values for ACE (in comparison with Sbt and Ctr) are higher, while, on the other hand, the D_{37} values are lower. Thus, the enhanced resistance of ACE at the initial stage of the process and the high threshold of inactivation are compensated by the weakening of protection under prolonged radiation.

For radiation-induced inactivation of the enzymes the presence or the absence of a buffer and (or) a salt, in particular NaCl, is important. The experiments carried out by us indicate, first, that the radiation stability of ACE, as a rule, increases with the increase in the concentration of phosphate-borate buffer from 0.01 *M* to 0.025 *M*, and, second, salt-free solutions of ACE at various concentrations (with or without buffer) are much more radiation-sensitive.

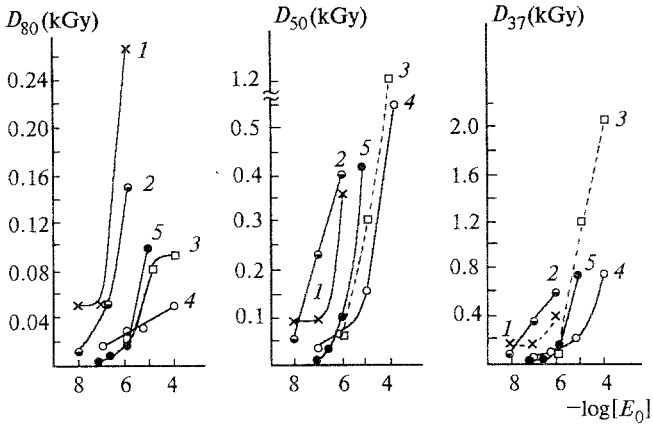

Fig. 4. Dependence of the dose D_{80}, D_{50}, and D_{37} on the initial concentration of enzymes: ACE (0.15 *M* NaCl): *1*, pH 9.0; *2*, pH 6.5; α-chymotrypsin: *3*, pH 7.8 (10^{-2} *M* Tris-HCl buffer); *4*, pH 3.0 (HCl); subtilysine-72: *5*, pH 5.5 (0.1 *M* NaCl, acetate buffer).

Table 1 lists the results of the study of the dose dependence of the inactivation of ACE in buffered and buffer-free media at different pH. These data attest to the stabilizing effect of the buffer on ACE at pH 6.5 and 9.0 at all stages of radiation-induced inactivation. However, at pH 7.5 the buffer destabilizes the enzyme at the initial stages of inactivation. This fact is clearly manifested in the D_{80} values and apparently reflects the conformational peculiarities of the ACE molecule in this pH range.

Experimental

Electrophoretically homogeneous ACE from bovine lungs,[13] and horseradish peroxidase (HRP) (Sigma, USA) were used. Furylacryloyl-*L*-phenylalanylglycylglycine (substrate **1**) (Sigma, USA) and carbobenzoxy-*L*-phenylalanyl-*L*-histidyl-*L*-leucine (substrate **2**) (Serva, FRG) were used as substrates for ACE. Determination of the enzymatic activity of ACE was performed spectrofluorometrically by following the rate of hydrolysis of substrate **1** ($7 \cdot 10^{-5}$ *M*) at 25 °C with a Shimadzu 256FW (Japan) spectrophotometer in 0.05 *M* buffer (HEPES, pH 7.5), containing 0.15 *M* NaCl and 1 μ*M* $ZnCl_2$[14] and also fluorometrically using tripeptide **2** as the substrate ($1 \cdot 10^{-5}$ *M*) in 0.05 *M* phosphate buffer (pH 7.5) containing 0.15 *M* NaCl.[15]

The enzymatic activity of HRP was determined spectrophotometrically at 405 nm, using ammonium 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonate) (substrate **3**).[16]

Irradiations were performed with a γ-source with P_γ = 0.05 Gy s^{-1} at 18—20 °C. Solutions of proteins (10^{-8}—10^{-6} *M*) for irradiation were prepared in a 0.025 *M* phosphate-borate buffer (containing or free from NaCl) or in 0.15 *M* NaCl. In the latter case, the pH of the solution was adjusted to the required value just before irradiation. After irradiation, the enzymatic activities of the samples were measured *vs.* the initial ones. No post-radiation effects were observed for ACE, but some were revealed for HRP. In this case, the results obtained were corrected in accordance with a post-radiation curve.

Table 1. Influence of a 0.025 *M* phosphate-borate buffer on the radiation-chemical parameters of inactivation of ACE*

pH	$G_{in} \cdot 10^3$	D_{80}	D_{50}	D_{37}
6.5	3	0.05	0.23	0.34
	1	0.17	0.75	0.6
7.5	2	0.15	0.41	0.52
	2	0.09	0.31	0.59
9.0	4.5	0.05	0.09	0.14
	1	0.31	0.38	0.5

Note. Conditions of inactivation: 10^{-7} *M*, 0.15 *M* NaCl, substrate **1**.
* The numerator gives the values obtained in the absence of buffer, and the denominator gives those in the presence of buffer.

Damage to 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 290.5 nm for tryptophan and 284.2 nm for tyrosine.[17] The spectrum of ACE (0.15 mg mL^{-1}) and its second derivative were obtained with a Shimadzu 265FW spectrophotometer. The measurements were carried out at 20 °C in the 300 to 275 nm wavelength range using a 1 nm slit width and the "Slow" scanning rate.

The radiation-chemical yield of inactivation (G_{in}) was calculated as the tangent to the dose curve without taking the induction period into account:

$$G_{in} = 0.96 \cdot 10^6 \, \Delta E/\Delta D, \qquad (1)$$

where G_{in} is the number of inactivated molecules per 100 eV of absorbed energy, E is the molar concentration of enzyme, and D is a dose of irradiation (kRad).

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