Radiation-induced inactivation of the angiotensin-converting enzyme in solutions
3.* The effect of NaCl

M. A. Orlova, O. A. Kost, I. I. Nikol'skaya, N. N. Troshina, and P. V. Binevski*

Department of Chemistry, M. V. Lomonosov Moscow State University, Leninskie Gory, 119899 Moscow, Russian Federation. Fax: +7 (095) 932 8881. E-mail: orlova@radio.chem. msu.ru

The effect of NaCl concentration (0—0.15 mol L^{-1}) and γ-irradiation dose on the catalytic activity of the angiotensin-converting enzyme is considered. Special regions in which a particular mechanism of dose response predominates are identified. In acid and alkaline media, there are regions of substantial enzymic activation; in addition, damped oscillations of the enzymic activity are observed. At pH 7.5, when the enzyme adopts a more "unfolded" conformation, the clearly defined activation peaks on the surface relief are smoothed over, indicating a decrease in the effect of the salt concentration.

Key words: radiation-induced inactivation, angiotensin-converting enzyme, sodium chloride, radiation-induced activation, γ-irradiation.

Previously, we studied the behavior of the angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) under various conditions using a radioenzymological method.[1,2] On the whole, the ACE exhibited relatively high radiation stability, which was manifested as the appearance of an induction (lag) period, whose duration increased upon increase in the enzyme concentration. The latent damages occurring during this period are accompanied by the destruction or modification of ~50% of the Trp and Tyr residues, which perform the autoprotector function in the molecule of this enzyme, together with the carbohydrate component.[2] However, it was noted that irradiation with low doses (starting from 0.2 Gy) resulted in enzymic activation; this makes the character of the dose response of the ACE molecule more complex than that for some other enzymes exposed to similar doses.[1,3] The activation effect can be manifested either as a primary or as a secondary effect, *i.e.*, one observed after a lag period and/or inactivation. The sum of the results obtained allowed us to claim[1] that the activation effect is manifested most clearly where the conformational flexibility of the active site is higher.

The observed dose response of the enzyme molecule (*i.e.*, the dependence of its catalytic activity on the irradiation dose) was pH- and enzyme concentration-dependent and included a lag period, an activation period, and consecutive activation and inactivation processes (oscillations of catalytic activity reflecting the conformational unbalance).[1] We proposed a mathematical model describing the dose response of an enzyme and demonstrated that conditions for the generation of damped oscillations of the enzymic activity on irradiation can exist.[4]

The dose response of an enzyme depends also on another important factor, namely, the presence of various effectors, including inorganic salts, in the solution. Thus our study[5] of the influence of calcium and magnesium salts on the radiation stability of plant peroxidases demonstrated the presence of both a specific influence of metal ions on the flexibility of the active site and the surface effect associated with the neutralization of the surface charges of the protein globule by metal cations.

Anions are known[6] to have a substantial influence on the catalytic activity of the ACE. Chloride anions are the most efficient activating species. They are believed to bind to the Lys residue near the active site of the enzyme.[6]

Our earlier experiments dealing with the behavior of the ACE on exposure to radiation were carried out using the physiological concentration of NaCl (0.15 mol L^{-1}). In this work, we studied the influence of NaCl in the concentration range from 0 to 0.15 mol L^{-1} on the dose response of the ACE at various pH; the results are presented as surfaces in the enzymic activity—absorbed dose—NaCl concentration coordinates. This way of presentation of experimental data in radioenzymology with allowance for several variable parameters permits one to identify clearly not only singular points but also the regions in which a particular mechanism of the dose response predominates and oscillations can be observed.

* For Part 2, see Ref. 1.

Translated from *Izvestiya Akademii Nauk. Seriya Khimicheskaya*, No. 12, pp. 2359—2363, December, 1999.

1066-5285/99/4812-2334 $22.00

Results and Discussion

Based on our earlier data[1] on the pH-dependence of the radiation-induced inactivation of the ACE in 0.15 *M* solutions of NaCl, one can claim that on passing from relatively low pH values (5.5) to high pH values (9.0), the conformation of the enzyme active site changes, namely, a fairly flexible structure is transformed into a more rigid one. The conformation transition is observed[1] at about pH 7.0—7.5. Therefore, to study the influence of NaCl on the properties of the ACE, we chose three pH values (6.0, 7.5, and 9.0) and two enzyme concentrations (10^{-8} and 10^{-7} mol L^{-1}); thus, we could estimate the change in the influence of the NaCl concentration on the properties of the enzyme under conditions where the flexibility of the active site changes. It should be taken into account that any change in the composition of the solution affects the ratio of the concentration of the reactive water radiolysis products to the concentration of enzyme molecules ($[R]/[E_0]$), which is a significant factor determining the possibility of enzymic activation and oscillations of the enzymic activity.

The processes occurring on irradiation of 10^{-8} *M* solutions of the ACE under conditions of variation of the above-mentioned parameters are summarized in Table 1. For the physiological pH value, 7.5, no primary activation was observed at any salt concentrations. Secondary activation (after inactivation) was noted only for low NaCl concentrations (≤0.005 mol L^{-1}); an increase in the salt concentration resulted in a plateau instead of secondary activation for the same doses. The physiological NaCl concentration (0.15 mol L^{-1}) gave only a lag period on the curve for the variation of the activity *vs* irradiation dose. As noted above, pH ≈ 7—7.5 is the pH range of the conformation transition[1] giving a more "unfolded" state, in which the accessible surface area of the globule is greater.

At other values of the pH of the medium, the curves for the catalytic activity *vs* irradiation dose exhibit primary activation, and the successive change in the salt concentration results in the alternation of processes (see Table 1), unlike the situation at pH 7.5, where the mechanism of the dose response of the ACE changes smoothly. Thus at pH 6.0, primary activation is observed for two salt concentrations, *viz.*, 0.001 and 0.15 mol L^{-1}, secondary activation is observed at a concentration of 0.05 mol L^{-1}, and the lag period is observed at 0.005 and 0.1 mol L^{-1}. At pH 9.0, primary activation takes place at NaCl concentrations of 0.01 and 0.1 mol L^{-1}, secondary activation occurs at a concentration of 0.005 mol L^{-1}, and the lag period is exhibited at concentrations of 0.05 and 0.15 mol L^{-1}.

Table 1. Formal description of processes occurring on γ-irradiation of 10^{-8} *M* solutions of the ACE at various pH values and NaCl concentrations

[NaCl] /mol L^{-1}	pH 6.0	pH 7.5	pH 9.0
0	in → pl → in	in → a → in	i → pl → in
0.001	a → in	in → a → in	i → pl → in
0.005	pl → in	in → a → in	→ a → in
0.01	in → pl → in	in → pl → in	a → in
0.05	in → a → in	in → pl → in	pl → in
0.10	pl → in	in → pl → in	a → in
0.15	a → in	pl → in	pl → in

Note. The following designations are used: in is inactivation, a is activation, pl is plateau.

These results imply that the flexibility of the active site changes depending on the medium and allow one to distinguish conditions under which a particular process clearly predominates. To identify the singular points in which one mechanism of the process is replaced by another, we presented the experimental results as surfaces (Fig. 1) in the coordinates "residual enzymic activity—absorbed dose—NaCl concentration" for the selected pH values. In this case, the singular points look like breaks on the surface relief. It should be noted that the relief of the surface (*i.e.*, the dose response pattern) observed for pH 6.0 and 9.0 is more complicated than that for pH 7.5, when the conformation of the protein globule has a greater accessible surface.

As was to be expected, the positions of the singular points are determined not only by characteristics of the solution (pH, salt concentration) but also by the dose, *i.e.*, it is related to the $[R]/[E_0]$ ratio. Thus, the positions of these points should depend also on the dose rate of the radiation source (this changes the initial $[R]/[E_0]$ ratio); however, this is the subject of a special investigation. The results obtained confirm that the surface of the ACE globule contains regions responsible for the conformational flexibility of the active site and the molecule as a whole, although they are not necessarily connected to the site itself. The amino acid composition of these regions should largely determine the dose response of the molecule and these regions should be capable of transferring energy or electron density ("mobile radical"). Therefore, the reactive water radiolysis products present in a substantial excess can induce an unbalance, resulting in the change in the accessible surface area of the active site due to oscillation processes in the molecule, even without influencing directly the active site. In this case, the absorbed dose can be small. We suggest that it is the Trp residues, which undergo intense modification in the initial steps of irradiation of the ACE,[2] that act as the key energy accumulators able to induce the enzymic activation and cause any energetic and, hence, conformational unbalance. This conclusion is entirely consistent with the literature data[7,8] on the role of Trp residues in the enzyme structure.

Table 2 presents the changes of the radiation-chemical parameters depending on the concentration of NaCl and pH of the medium. The radiation-chemical yield of

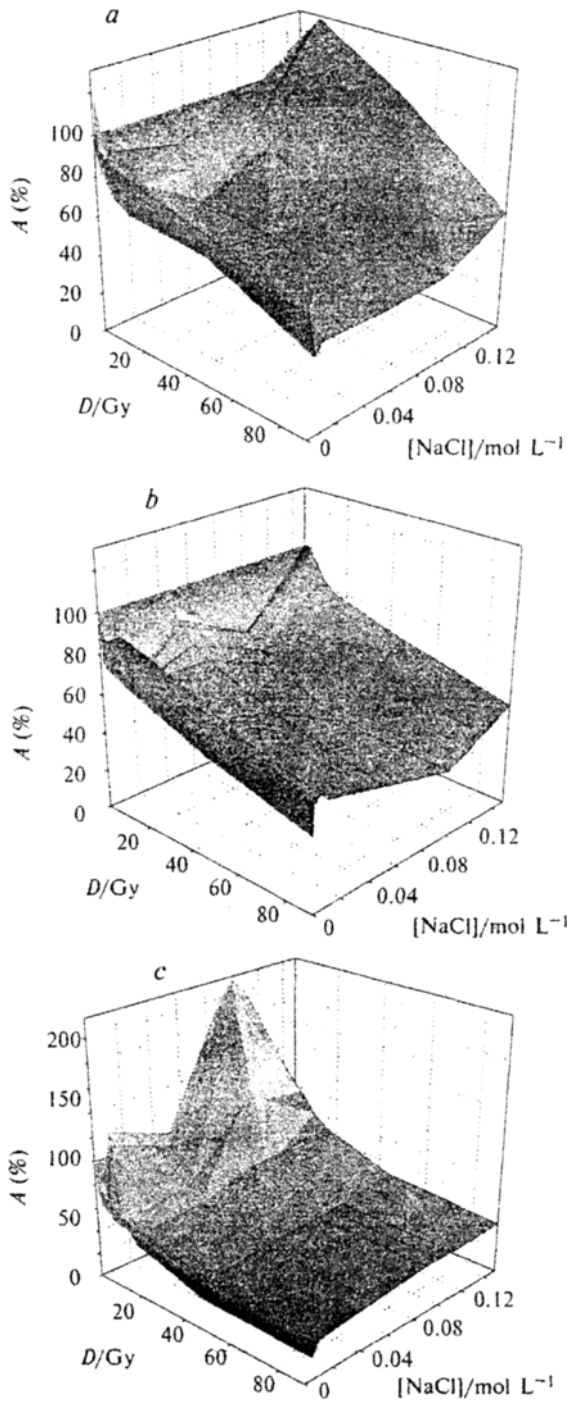

Fig. 1. Dose response of a 10^{-8} *M* solution of the ACE at various concentrations of NaCl and pH = 6.0 (*a*), 7.5 (*b*), 9.0 (*c*); *A* is activity, *D* is irradiation dose.

inactivation G_{in} is the most important parameter. The salt concentration affects appreciably the G_{in} value; the maximum inactivation rate (increase in G_{in}) is observed at NaCl concentrations of 0.05 mol L^{-1} (at pH 6.0), 0.01 mol L^{-1} (at pH 7.5), or 0.1 mol L^{-1} (at pH 9.0).

To charac rize the period of possible unbalance in the molecule nd hence, to some extent, its margin of safety, we int oduced the parameter D_{dl} (the dose after which only ir versible inactivation is observed). The D_{dl} values (see T ble 2) are pH-dependent (when the salt concentratio is constant) and salt concentration-dependent (wh the pH is constant). For 10^{-8} *M* solutions of the A E, an increase in the period of unbalance is often acc mpanied by an increase in the rate of subsequent i ctivation of the enzyme.

At pH 6. , the addition of the salt to the system causes a sub antial drop of D_{dl}; further increase in the salt concentr ion results in a small increase in the D_{dl} value. At pH .5 and 9.0, the dependence of D_{dl} on the salt concentr ion passes through maxima at NaCl concentrations c 0.001 and 0.01 mol L^{-1} (pH 7.5) and at a NaCl conce ration of 0.001 mol L^{-1} (pH 9.0). This provides an additional illustration of the difference between the dc responses and, hence, the conformations of the enzyn molecule at various pH values. It should be noted tha change in the parameter D_{dl} can be related in some cas to change in the ionic strength of the solution (Tal e 3); however, the changes in D_{dl} observed at low salt c ncentrations (0—0.01 mol L^{-1}) occur at virtually con ant μ.

To descr e the dose response in more detail, we present (see able 2) parameters which characterize the latent dama (D_{ind}), inactivation (D_{in} and Δ_{in}), and activation (D_a and Δ_a) processes during the conformational unbala ce period.

As has be n reported previously,[1,2] an increase in the ACE concen ration changes the pattern of the enzyme dose respons due to enhanced competition between various proc sses as a result of the decrease in the $[R]/[E_0]$ rati Therefore, variation of the concentration of NaCl cha ges all the radiation-chemical parameters, as shown in able 3.

Comparis n of the observed dose responses of the ACE at pH 7 for different enzyme concentrations (10^{-8} and 10^{-7} mo L^{-1}, see Tables 1—3) shows that a sort of a "shift" alo g the dose response vector occurs. For example, wh n the concentration of NaCl varies from 0 to 0.005 mol L^{-1}, a transition from a 10^{-8} *M* solution of the ACE to 10^{-7} *M* solution switches the "inactivation—activat n—inactivation" process to the "activation—inactiv ion" process. As the salt concentration increases (0. 05—0.01 mol L^{-1}), the variations of the G_{in} values fo both ACE concentrations follow a general pattern, *i.e.*, both dependences have one maximum. However, th duration of the period of unbalance in the more conce rated enzyme solution depends on the NaCl concer ration to a lesser extent.

Thus, the influence of NaCl on the functioning of the ACE is s bstantial and manifests itself as enhancement or we kening of the conformational unbalance upon variati n of the conditions in the medium. This, in turn, gives r se to clearly defined singular points (regions) on th surface for the variation of the enzymic

Table 2. Radiation-chemical parameters for irradiation of 10^{-8} *M* solutions of the ACE at various pH values and NaCl concentrations

[NaCl] /mol L^{-1}	pH	$G_{in} \cdot 10^3$	D_{ind}	D_a	Δ_a (%)	D_{dl}	D_{in}	Δ_{in} (%)
			Gy			Gy		
0	6.0	3.2	—	—	—	60	15	35
	7.5	0.8	—	3	12	3	1.5	25
	9.0	0.3	—	—	—	4	1.5	25
0.001	6.0	0.5	—	1	16	1	—	10
	7.5	0.4	—	9	12	9	1.5	20
	9.0	0.1	—	—	—	15	1.5	30
0.005	6.0	0.5	9	—	—	9	—	—
	7.5	0.7	—	3	18	3	0.5	30
	9.0	0.6	—	3	30	3	1.5	30
0.01	6.0	0.6	—	—	—	10	3	15
	7.5	3.2	—	—	—	30	0.5	30
	9.0	0.1	—	0.5	20	0.5	—	—
0.05	6.0	2.0	—	3	15	3	1.5	30
	7.5	0.8	—	—	—	15	0.5	15
	9.0	0.9	1	—	—	1	—	—
0.10	6.0	1.5	—	—	—	3	—	—
	7.5	0.8	—	—	—	3	0.5	30
	9.0	1.7	—	0.5	12	0.5	—	—
0.15	6.0	1.2	—	9	30	9	—	—
	7.5	1.8	3	—	—	3	—	—
	9.0	1.6	3	—	—	3	—	—

Note. G_{in} is the radiation-chemical yield of inactivation determined using the section on the dose response curve after D_{dl} (the number of inactivated molecules per 100 eV of absorbed energy); D_{ind} is the dose up to which the induction (lag) period is observed; D_{dl} is the dose after which only inactivation is observed; D_a is the dose at which the maximum activation is attained; Δ_a is the percent by which the enzymic activity at the maximum activation exceeds that at the maximum inactivation or that at the plateau; D_{in} is the dose at which the inactivation maximum during the conformational unbalance period is observed; Δ_{in} is the percent by which the enzymic activity has decreased in the conformational unbalance region with respect to the initial activity. The experimental error is ~10%.

Table 3. Change in the radiation-chemical parameters for γ-irradiation of a 10^{-7} *M* solution of the ACE at various NaCl concentrations (pH 7.5)

[NaCl] /mol L^{-1}	μ^b	$G_{in} \cdot 10^3$	D_{dl}	D_a	Δ_a (%)	D_{ind}	D_{in}	Δ_{in} (%)	Description of the process[c]
			Gy			Gy			
0	2.0	10	3	3	45	—	—	—	a → in
0.001	2.1	15	15	15	75	—	—	—	a → in
0.005	2.2	60	15	15	20	—	—	—	a → in
0.01	2.4	15	10	—	—	10	—	—	pl → in
0.05	4.4	10	10	—	—	—	0.5	40	in → pl → in
0.10	6.5	10	10	—	—	—	0.5	40	in → pl → in
0.15	7.2	10	3	3	20	0.5	—	—	pl → a → in

[a] See Note to Table 2.
[b] Ionic strength of the solution.
[c] See Note to Table 1.

activity as a function of the absorbed dose and the concentration of NaCl and results in more clear-cut oscillations of enzymic activity.

Experimental

Electrophoretically homogeneous ACE from bovine lungs was isolated and purified as described previously.[9] The enzymic activity was determined by fluorimetry[1] using Z-Phe-His-Leu as the substrate (Serva). Solutions were irradiated by a γ-source with a dose rate P_γ = 0.05 Gy s^{-1} at ~20 °C. Solutions of the protein (with concentrations of 10^{-8} and 10^{-7} mol L^{-1}) were prepared in a 0.025 *M* phosphate–borate buffer at an appropriate pH. The concentration of NaCl was varied in the 0—0.15 mol L^{-1} range. After irradiation, the solutions were kept for 1 h to let possible secondary reactions come to completion. No post-radiation effects were observed for the ACE.

The radiation-chemical yields of inactivation (G_{in}) (change in the enzymic activity per 100 eV of absorbed energy) were calculated from the section on the dose response curve that immediately follows the attainment of the D_{dl} point using the equation

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

where ΔE/mol L^{-1} is the change in the enzyme concentration, and ΔD/krad is the change in the dose.

References

1. O. A. Kost, M. A. Orlova, N. N. Troshina, and I. I. Nikol'skaya, *Izv. Akad. Nauk, Ser. Khim.*, 1996, 223 [*Russ. Chem. Bull.*, 1996, **45**, 215 (Engl. Transl.)].
2. M. A. Orlova, O. A. Kost, I. I. Nikol'skaya, N. N. Troshina, and A. A. Shevchenko, *Izv. Akad. Nauk, Ser. Khim.*, 1994, 1322 [*Russ. Chem. Bull.*, 1994, **43**, 1260 (Engl. Transl.)].
3. M. A. Orlova, *Izv. Akad. Nauk, Ser. Khim.*, 1996, 2828 [*Russ. Chem. Bull.*, 1996, **45**, 2679 (Engl. Transl.)].
4. M. A. Orlova and V. A. Egorov, *Izv. Akad. Nauk, Ser. Khim.*, 1999, 664 [*Russ. Chem. Bull.*, 1999, **48**, 659 (Engl. Transl.)].
5. M. A. Orlova, T. A. Chubar', V. A. Fechina, and I. G. Gazaryan, *Izv. Akad. Nauk, Ser. Khim.*, 1998, 522 [*Russ. Chem. Bull.*, 1998, **47**, 505 (Engl. Transl.)].
6. P. Corvol, T. A. Williams, and F. Soubrier, *Methods Enzymol.*, 1995, **248**, 283.
7. N. V. Timofeev-Resovskii, A. V. Savich, and M. I. Shal'nov, *Vvedenie v molekulyarnuyu radiobiologiyu* [*Introduction to Molecular Radiobiology*], Meditsina, Moscow, 1981, 193 pp. (in Russian).
8. A. G. Bezrukova and I. Yu. Ostashevsky, *Int. J. Rad. Biol.*, 1977, **31**, 131.
9. T. Orth, S. Voronov, P. Binevski, W. Saenger, and O. A. Kost, *FEBS Lett.*, 1998, **431**, 255.

Received June 15, 1999