Regulatory proteins (inhibitors or activators) affect estimates of M_r of enzymes and receptors by radiation inactivation

A theoretical model

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The radiation-inactivation method allows the determination of the M_r of enzymes and receptors by monitoring the decay of biological activity as a function of absorbed dose. The presence of regulatory or effector proteins (inhibitors or activators) associated with an enzyme or receptor, or released in the preparation after tissue homogenization, may affect the decay of biological activity. How the activity is affected, however, will depend on the type of inhibition (competitive or non-competitive), the inhibitor or activator concentration, the dissociation constant of the enzyme-effector system, and the effector M_r relative to that of the enzyme. Since little is known on how effector proteins influence radiation inactivation of enzymes and receptors, we have considered a theoretical model in an effort to provide a framework for the interpretation of experimentally obtained data. Our model predicts that competitive and non-competitive inhibitors of enzymes could be distinguished by analysing irradiated samples with various substrate concentrations. Inhibitors will decrease whereas activators will increase the apparent target size of enzymes or receptors.

The radiation-inactivation method is widely used to determine M_r (target size) of membrane enzymes and receptors *in situ* (Kepner & Macey, 1968; Levinson & Ellory, 1974; Kempner & Schlegel, 1979). Crude preparations of freeze-dried or frozen solutions of proteins are irradiated directly without purification or solubilization, and biological activity is determined as a function of absorbed dose, after rehydration or thawing. The principle of the radiation-inactivation method is based on the assumption that a single hit of an ionizing radiation on a macromolecule will destroy completely its biological activity (Lea, 1955). The target theory predicts that radiation exposure will lead to biological activity decay as a single exponential function of the absorbed dose:

$$v_D = v_0 e^{-\mu D} \quad (1)$$

where v_0 is the enzyme activity at zero dose and v_D is the enzyme activity after an absorbed dose D . The coefficient μ is directly proportional to the M_r

of the enzyme and represents the slope of the semi-logarithmic plot of remaining enzyme activity versus absorbed dose.

Regulatory proteins, physically associated with enzymes or receptors, may interfere with the determination of biological activity in irradiated preparations and yield anomalous radiation-inactivation curves. In addition, inhibitors or activators released by the homogenization procedure may interact with the enzyme or receptor of interest in crude preparations. Simon *et al.* (1982) have described a general mathematical model simulating radiation inactivation of an equilibrium regulatory-protein-enzyme system. In the present paper we describe a particular application of this general model concerning inhibitors and activators.

Harmon *et al.* (1980) have described the radiation inactivation of the regulated insulin receptor of rat liver plasma membranes. These authors observed increased insulin binding at low radiation doses and suggested that this unexpected effect was due to the presence of a large- M_r regulatory (inhibitory) protein closely associated with the receptor in the membrane. Since the

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target size of this inhibitory protein (M_r 350000) was greater than that of the receptor (M_r 87000), it was inactivated first under radiation exposure, thus explaining increased binding by the receptor at low radiation doses. Increasing the insulin concentration in the frozen receptor preparation during irradiation was reported by these authors to normalize the radiation-inactivation curve by diminishing the effect of the regulatory component (Harmon *et al.*, 1980, 1981). They also mentioned in their paper that an artificial mixture of α_2 -macroglobulin and trypsin also behaved like the insulin receptor, trypsin activity increasing at low radiation doses. From the equations of competitive and non-competitive enzyme inhibitions, we propose a model that allows one to draw theoretical radiation-inactivation curves for enzymes irradiated in the presence of large- M_r inhibitors, and we define the conditions necessary to obtain an increase of biological activity at low radiation doses. A similar development was made for activators.

transfer between the regulatory unit and enzyme has been considered previously by Simon *et al.* (1982), and the equations that we propose could be modified to take into account the fraction of bound enzyme and regulatory protein that may be present during radiation exposure.

The decay of enzyme and inhibitor as a function of absorbed dose is described by the following two equations:

$$E_D = E_0 e^{-\mu_E D} \quad (2)$$

$$I_D = I_0 e^{-\mu_I D} \quad (3)$$

where E_D and I_D represent the quantity of enzyme and inhibitor respectively after an absorbed dose D , E_0 and I_0 are the initial quantities of enzyme and inhibitor before irradiation, and μ_E and μ_I are the decay coefficients, proportional to the M_r of enzyme and inhibitor respectively.

When eqns. (2) and (3) are substituted for enzyme and inhibitor concentrations in the fundamental equations of inhibition, we obtain:

$$v_D = \frac{kE_0 e^{-\mu_E D}}{1 + \frac{K_m}{S} \left(1 + \frac{I_f}{K_i} e^{-\mu_I D} \right)} \quad (\text{competitive inhibition}) \quad (4)$$

where

$$I_f = \frac{1}{2} \{ -K_i (1 + S/K_m) - E_0 e^{-\mu_E D} + I_0 e^{-\mu_I D} + [(K_i (1 + S/K_m) + E_0 e^{-\mu_E D} - I_0 e^{-\mu_I D})^2 + 4K_i (1 + S/K_m) I_0 e^{-\mu_I D}]^{1/2} \} \quad (5)$$

$$v_D = \frac{kE_0 e^{-\mu_E D}}{\left(1 + \frac{K_m}{S} \right) \left(1 + \frac{I_f}{K_i} e^{-\mu_I D} \right)} \quad (\text{non-competitive inhibition}) \quad (6)$$

where

$$I_f = \frac{1}{2} \{ -K_i - E_0 e^{-\mu_E D} + I_0 e^{-\mu_I D} + [(K_i + E_0 e^{-\mu_E D} - I_0 e^{-\mu_I D})^2 + 4K_i \cdot I_0 e^{-\mu_I D}]^{1/2} \} \quad (7)$$

Theory and results

Competitive and non-competitive inhibitors

Theoretical treatment. The following assumptions were made: (1) according to target theory (Lea, 1955), the irradiated inhibitor loses biological activity as an exponential function of absorbed dose; (2) the K_m and K_i values of the enzyme and inhibitor respectively are unchanged by radiation exposure; (3) the equilibrium between concentrations of free enzyme, inhibitor and the complex of the two molecules is always reached in the incubation medium used for enzyme assay after irradiation; (4) the transfer of absorbed radiation energy between the enzyme and inhibitor molecules is negligible. The possibility of energy

v_D is the velocity of the enzyme reaction after an absorbed dose D , k is the catalytic constant of the enzyme, S is the substrate concentration in the assay medium, I_f is the free inhibitor concentration, K_m is the Michaelis-Menten constant and K_i is the inhibition constant. Eqns. (4) and (6) were used to generate, with a computer, a series of theoretical radiation-inactivation curves corresponding to various μ_I values (Fig. 1). The parameters kE_0 , K_m/S , I_0 and K_i in eqns. (4) and (6) were arbitrarily set to a value of 1 and μ_E to 0.1. A μ_E value of 0.1 corresponds to an M_r of 64000. It must be pointed out, however, that it is the ratio of μ_I to μ_E that affects the shape of the curves presented in Fig. 1.

For μ_I values lower than μ_E , the presence of the

Fig. 1. Simulation of radiation inactivation of a competitive-inhibitor-enzyme system

The eqn. (4) is used to generate the theoretical curves. Parameter values are $kE_0 = I_0 = K_i = K_m/S = 1$ and $\mu_E = 0.1$. The μ_i value is equal to 0 (curve 1), 0.03 (curve 2), 0.1 (curve 3), 0.3 (curve 4) and 1 (curve 5).

Fig. 2. Computer-generated radiation-inactivation curves of a competitive-inhibitor-enzyme system (a) and a non-competitive-inhibitor-enzyme system (b) at various substrate concentrations

All parameter values of eqns. (4) and (6) are as in the legend to Fig. 1 except that μ_i is 1 and K_m/S is 0 (curve 1), 0.3 (curve 2), 0.5 (curve 3), 1 (curve 4), and 5 (curve 5).

inhibitor introduces an error in the determination of the M_r (apparent lower value) and causes a curvature in the plot of v_D versus D (Fig. 1). This curvature may not be apparent from experimentally obtained data, considering that, in practice, experimental error in enzyme determinations may be around 10%. For μ_i values larger than μ_E , the enzyme activity increases at low radiation doses owing to preferential inactivation of the larger-target-size inhibitor.

Consequences of the theoretical model. The model predicts that it is possible to distinguish between competitive and non-competitive types of inhibition by assaying enzyme activity, after irradiation, by using different substrate concentrations in the incubation medium (Fig. 2). In the case of competitive inhibition, the dose at maximum enzyme activity (D_{max}) will decrease with increasing substrate concentration (Fig. 2a), whereas in the non-competitive inhibition D_{max} will be independent of substrate concentration (Fig. 2b). The family of curves obtained in Fig. 2(a) resemble those obtained by Harmon *et al.* (1980, 1981) for the insulin receptor in the presence of increasing insulin concentrations.

Finally, the effect of varying concentrations of inhibitors on the radiation-inactivation curves of an enzyme is shown in Fig. 3. For high concentrations of inhibitor (relative to K_i) and μ_i much larger than μ_E , the enzyme activity increases at low radiation doses. Similar shapes of curves were obtained for both competitive and non-competitive types of inhibition.

Activators

Theoretical treatment. The assumptions made for enzyme-inhibitor interactions were also used for

the enzyme-activator system. In summary: (1) it was assumed that the activator loses activity as an exponential function of absorbed dose; (2) K_A , the activation constant, and K_m are unaffected by radiation exposure; (3) the equilibrium concentrations between enzyme, activator and substrate are reached during enzyme assay after irradiation; (4) the transfer of energy between enzyme and activator is negligible. The equation for v_D is:

$$v_D = \frac{kE_0 e^{-\mu_E D}}{\left(1 + \frac{K_m}{S}\right) \left(1 + \frac{K_A}{A_f e^{-\mu_A D}}\right)} \quad (8)$$

where

$$A_f = \frac{1}{2} \left\{ -K_A - E_0 e^{-\mu_E D} + A_0 e^{-\mu_A D} + \left[(K_A + E_0 e^{-\mu_E D} - A_0 e^{-\mu_A D})^2 + 4K_A \cdot A_0 e^{-\mu_A D} \right]^{1/2} \right\} \quad (9)$$

A_0 is the initial activator concentration before irradiation and K_A is the enzyme-activator dis-

Fig. 3. Computer-generated radiation-inactivation curves of a competitive-inhibitor-enzyme system or a non-competitive-inhibitor-enzyme system at various inhibitor concentrations

All parameter values are as in the legend to Fig. 1 except that μ_i is 1 and I_0 is 0.01 (curve 1), 0.1 (curve 2), 1 (curve 3), 10 (curve 4) and 100 (curve 5).

Fig. 4. Simulation of radiation inactivation of an activator-enzyme system

The eqn. (8) is used to generate the theoretical curves. Parameter values are $kE_0 = K_A = A_0 = K_m/S = 1$ and $\mu_E = 0.1$. The μ_A value is equal to 0 (curve 1), 0.03 (curve 2), 0.1 (curve 3), 0.3 (curve 4) and 1 (curve 5).

sociation constant. The theoretical radiation inactivation curves generated with eqn. (8), corresponding to different μ_A values, indicate that the presence of large- M_r activators in the enzyme preparation would increase the apparent M_r of the enzyme (Fig. 4). Varying the K_A and A_0 values gives a series of radiation-inactivation curves with downward curvatures at low K_A relative to A_0 values (Fig. 5).

Discussion

Harmon *et al.* (1980, 1981) reported an increase in insulin-receptor affinity for and binding of

Fig. 5. Computer-generated radiation-inactivation curves of an activator-enzyme system at various activator concentrations

All parameter values of eqn. (8) are as in the legend to Fig. 4 except that μ_A is 0.5 and K_A is 100 (curve 1), 10 (curve 2), 1 (curve 3), 0.1 (curve 4) and 0.01 (curve 5).

insulin at low radiation doses that was attributed to the presence of a large- M_r inhibitor associated to the receptor. These authors also observed a similar effect with trypsin in an artificial mixture with α_2 -macroglobulin *in vitro*. Simon *et al.* (1982) have proposed a general mathematical model describing radiation inactivation of regulated enzymes and receptors, but they have made no distinction between different types of inhibitory effectors. In the present paper we have used the equations of competitive and non-competitive types of inhibition to predict the shape of radiation-inactivation curves of enzymes and receptors.

The results of our theoretical considerations indicate that the presence of macromolecular inhibitors or activators may cause erroneous estimates of M_r of enzymes and receptors by radiation inactivation. This must be kept in mind during analysis of radiation-inactivation data obtained with crude preparations, which may contain inhibitors or activators. On the other hand, in well-defined systems, such as regulated membrane-bound enzymes and receptors in partially purified preparations, the analysis of radiation-inactivation curves obtained at different concentrations of substrate (for enzymes) or ligand (for receptors) may yield information on the type of inhibition present (competitive or non-competitive).

Some of the assumptions made to construct the theoretical model are experimentally testable. First, it was assumed that effectors (inhibitors and activators) are inactivated in accordance with the single-hit inactivation model of target theory, i.e. as an exponential function of absorbed dose (Lea, 1955). Secondly, the K_m and K_i values of the

enzyme and inhibitor (K_A for an activator) respectively were assumed to be unaffected during the course of radiation exposure. This is also deduced from the basic assumptions of target theory, i.e. that inactivation is an all-or-none mechanism; each molecule hit by an ionizing radiation loses its biological activity completely. The dissociation constant of several receptors for their respective ligands is unchanged by radiation exposure even when most of the molecules have been destroyed (Kempner & Schlegel, 1979). In some well-defined systems, such as a mixture of α_2 -macroglobulin and trypsin (Harmon *et al.*, 1980), this assumption would be verifiable. Thirdly, it was assumed that the equilibrium is always reached between concentrations of enzyme, inhibitor, substrate and the corresponding complexes in the assay medium used after irradiation to determine enzyme activity, and that the inactivated enzyme molecule does not bind the inhibitor. This assumption may be difficult to test, but sufficient time should be allowed after reconstitution (rehydration or thawing) to reach equilibrium. Finally, it was hypothesized that there was no energy transfer between the components of the complex. This can be verified in a purified enzyme-inhibitor system by measuring the quantity of each component by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as a function of radiation dose. However, energy transfer has been demonstrated only between subunits of some enzymes (Kempner & Schlegel, 1979; Saccomani *et al.*, 1981) and not between heterologous proteins.

From an experimental point of view, it is important to use both very high and low substrate concentrations in order to get a good discrimination between the curves shown in Fig. 2. The experimental error should also be diminished as much as possible in the assay system.

In conclusion, the radiation inactivation of an enzyme or receptor, in the presence of an inhibitor or an activator, may yield erroneous estimates of M_r . However, in well-defined systems, we believe that, by using the theoretical basis presented in this paper, the radiation-inactivation method could yield valuable information on the nature of the inhibitor and how its presence affects the target size of enzyme or receptor. The mathematical model was especially developed to study radiation-inactivation curves of enzyme-inhibitor systems that show an increase in biological activity at low radiation doses. The conditions necessary to obtain such curves were clearly defined.

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