Size determination of an equilibrium enzymic system by radiation inactivation

Theoretical considerations

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Radiation inactivation of complex enzymic systems is currently used to determine the enzyme size and the molecular organization of the components in the system. We have simulated an equilibrium model describing the regulation of enzyme activity by association of the enzyme with a regulatory unit. It is assumed that, after irradiation, the system equilibrates before the enzyme activity is assayed. Our theoretical results show that the target-size analysis of these numerical data leads to a bad estimate of the enzyme size. Moreover, some implicit assumptions such as the transfer of radiation energy between non-covalently bound molecules should be verified before interpretation of target-size analysis. It is demonstrated that the apparent target size depends on the parameters of the system, namely the size and the concentration of the components, the equilibrium constant, the relative activities of free enzyme and enzymic complex, the existence of energy transfer, and the distribution of the components between free and bound forms during the irradiation.

Since the 1950s, enzyme inactivation by irradiation has been widely used for determining the size of enzyme molecules. This method does not require the solubilization or the purification of membrane-bound enzymes. Therefore the interpretation of the experimental data by the target-size analysis allows characterization of the enzyme size *in situ* (for a review see Kempner & Schlegel, 1979).

Target-size analysis is based on the fact that the decrease of enzyme activity due to the irradiation is a mono-exponential function of the radiation dose:

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

where v_0 is the enzyme activity before the irradiation and v_D is the activity after the exposition of the enzyme to the radiation dose D . μ is a coefficient that is proportional to the size of the enzyme. Eqn. (1) can be rewritten as follows:

$$\ln \left(\frac{v_D}{v_0} \right) = -\mu D \quad (2)$$

Thus μ can be determined as the negative slope of the curve in the linear plot of $\ln(v_D/v_0)$ versus D .

The physiological regulation of enzyme activity involves, in some cases, other molecules that interact

with the enzyme. Since the apparent size of the enzyme is increased when regulatory molecules are coupled with the enzyme, it has been admitted that target-size analysis could give more insight into the molecular organization of complex systems such as, for instance, the adenylate cyclase system (Houslay *et al.*, 1977; Martin *et al.*, 1979; Schlegel *et al.*, 1979; Nielsen *et al.*, 1981).

Target-size analysis has been used with success for the study of many enzymes, but the validity of the method has not been demonstrated for more complex systems, and some interpretations of its results have been questioned (Swillens & Dumont, 1981).

The aim of the present paper is to predict, by theoretical simulations, the characteristics of a complex system as obtained by target-size analysis. The equilibrium model studied in this work consists of the regulation of an enzyme E through the interaction with a regulatory unit R:

By using this model, data were generated and analysed in accordance with target-size analysis as it would have been applied to experimental data. Our theoretical simulations show that, generally, the decay curves of $\ln(v_D/v_0)$ plotted versus D obtained

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with this model are not linear, but, if a random error as low as 5% is allowed for each data point, statistical test cannot reject the apparent linearity. Consequently, an apparent size of the target might be evaluated. However, in this case, the calculated size does not reflect the actual size of the target. In fact, the size depends on the characteristics of the system, namely the component concentrations, the equilibrium constant, the relative activities of free enzyme E and of the complex RE, and the possible transfer of radiation energy between subunits when they are coupled.

Changes in apparent target size due to some experimental treatment have been attributed to a modification of the coupling state between the components (Houslay *et al.*, 1977; Martin *et al.*, 1979). Our theoretical results suggest that other simple explanations can be invoked, for instance a modification of the equilibrium constant due to the treatment. It thus appears that target-size analysis applied to complex equilibrium systems is generally neither capable of rejecting the validity of a proposed model, nor useful for estimating the actual target size, nor able to discriminate between structural and functional alterations of the system due to any treatment affecting the apparent target size. However, these conclusions should be reconsidered for each particular experimental system.

Model and methods

Definition of the model

The regulatory-and-catalytic-units model is defined by the equilibrium interactions between the regulatory unit R and the catalytic unit E:

where K_d is the dissociation equilibrium constant.

At equilibrium, the component concentrations obey the following equations:

$$K_d = \frac{[R][E]}{[RE]} \quad (3)$$

$$[E_t] = [E] + [RE] \quad (4)$$

$$[R_t] = [R] + [RE] \quad (5)$$

where $[R_t]$ and $[E_t]$ are the total concentrations of regulatory and catalytic units respectively. By using eqns. (3), (4) and (5) we get:

$$[RE] = \frac{1}{2} \left([R_t] + [E_t] + K_d - \sqrt{([R_t] + [E_t] + K_d)^2 - 4[R_t][E_t]} \right) \quad (6)$$

$$[E] = [E_t] - [RE] \quad (7)$$

$$[R] = [R_t] - [RE] \quad (8)$$

The initial velocity of the enzymic reaction is the sum of the respective contributions made by E and RE, i.e.:

$$v = (k_{cat})_E[E] + (k_{cat})_{RE}[RE] \quad (9)$$

Simulation of radiation inactivation

If it is assumed that radiation energy is not transferred from one unit to the other one within the complex RE, the total concentrations of R and E are mono-exponential functions of the radiation dose D :

$$[E_t]_D = [E_t]_0 \cdot e^{-\mu_E D} \quad (10)$$

$$[R_t]_D = [R_t]_0 \cdot e^{-\mu_R D} \quad (11)$$

where μ_E and μ_R are proportional to the size of E and R respectively. $[E_t]_0$ and $[R_t]_0$ are the total concentrations of E and R before irradiation.

If energy transfer occurs within the complex, and if association and dissociation of the components do not occur during the irradiation (low-temperature, freeze-dried or dried samples), then:

$$[E_t]_D = [E_t]_0 (f_{E_f} \cdot e^{-\mu_E D} + f_{E_b} \cdot e^{-(\mu_E + \mu_R) D}) \quad (12)$$

$$[R_t]_D = [R_t]_0 (f_{R_f} \cdot e^{-\mu_R D} + f_{R_b} \cdot e^{-(\mu_E + \mu_R) D}) \quad (13)$$

where f_{E_f} , f_{R_f} , f_{E_b} and f_{R_b} are respectively the fractions of free E and R and bound E and R at the beginning of the irradiation, i.e. $f_{E_f} + f_{E_b} = 1$ and $f_{R_f} + f_{R_b} = 1$.

Depending on the assumption made, the equation pair (10) and (11) or (12) and (13) is introduced in eqns. (6), (7) and (8), and v_D is computed (eqn. 9).

In some simulations, a random error is introduced by generating a random number issued from a normally distributed population with v_D as the mean and with a dispersion characterized by a 5% coefficient of variation.

Target-size analysis

Eleven data pairs (D , v_D) are generated with D varying with a constant step from zero to the dose leading to a 5% residual activity. Data points are plotted in the ' $\ln(v_D/v_0)$ -versus- D ' representation, and a straight line passing through the first point (zero dose) is fitted by linear regression according to the methodology currently used (see, e.g., Martin *et al.*, 1979). Statistically acceptable linear regression allows the determination of the relative target size as the absolute value of the slope of the straight line.

Statistical analysis

In order to check whether a systematic divergence exists between the straight line fitted by linear regression and the data plotted in the ' $\ln(v_D/v_0)$ -versus- D ' representation, a runs test is performed on the residuals (Siegel, 1956). The null hypothesis assumes that the signs of the residuals occur in random order. The null hypothesis will be rejected by the runs test at the 0.05 level of

significance. In this case, it is concluded that the data significantly deviate from linearity, i.e. v_D does not obey eqn. (1).

Results

The regulatory-and-catalytic-units model was simulated with the assumption that the enzymic activity is expressed only by the RE complex and that the radiation energy is not transferred between the subunits of the complex. The parameter values were $K_d = 1$, $[R_t] = [E_t] = 1$ and $\mu_E = \mu_R = 1$. Fig. 1 shows the activity decay curve and the slope of the curve as a function of the dose D . The theoretical curve is not linear, as shown by the non-constant slope, which varies between -1.48 and -2 . It appears that the size of the active entity RE is given by the absolute value of the slope at an infinitely high dose.

In order to mimic a realistic experiment, the same model has been simulated with eleven dose values ranging from 0 to 1.85. This latter value corresponds to a 5% residual activity, which is a commonly used limit in such experiments. A random error corresponding to a 5% coefficient of variation was introduced for each activity values (see the Model and methods section). A typical theoretical result is shown in Fig. 2(a). The straight line fitted by linear regression is characterized by a slope of -1.58 . The runs test applied to the signs of the residuals indicates that the data do not significantly deviate from linearity. This simulation has

Fig. 1. Simulation of the radiation inactivation of the regulatory-and-catalytic-units model

The logarithm of the ratio between residual activity v_D and control activity v_0 [$\ln(v_D/v_0)$] is plotted as a function of the radiation dose D (—). The first derivative of this function with respect to D is also represented as the slope (---). It is assumed that free E is inactive and that radiation energy is not transferred between R and E. The parameter values are $K_d = 1$, $[R_t] = [E_t] = 1$ and $\mu_E = \mu_R = 1$.

been done seven times. If these simulations actually represented seven experiments, it would be concluded that the simple mono-exponential model can describe the data and that the studied system would be characterized by an apparent target size of 1.57 ± 0.027 (mean $\pm \sigma$).

Other simulations were performed on the basis of the same model but with different parameter values and alternative hypotheses. Since the runs test generally does not reject the hypothesis that the data generated by this model with a random error of 5–10% obey eqn. (1), the next simulations do not introduce random errors. Thus the changes in the apparent target size will be due only to the variations of the model parameters.

Fig. 2. Target-size analysis of computer-generated data. Model parameter values and hypotheses are the same as in Fig. 1. A random error of 5% has been allowed for each data point. (a) Data points fitted by linear regression. The slope is equal to -1.58 . (b) Plot of the residuals versus dose:

$$R = 100 \times \frac{v_D^{\text{data}} - v_D^{\text{regr.}}}{v_D^{\text{regr.}}}$$

where v_D^{data} is the pseudo-experimental value and $v_D^{\text{regr.}}$ is the theoretical value obtained by linear regression.

The parameters affecting the equilibrium state of the system are the dissociation constant and the total concentrations of R and E. Fig. 3 shows how the apparent target size is sensitive to the ratios $[R_t]/[E_t]$ and $K_d/[E_t]$. It appears that the size varies between the size of the dissociated components and the size of the complex. For instance, for $[R_t]/[E_t] = 1$ and $K_d/[E_t] = 1$, the apparent size is equal to 1.58. Assuming that an experimental treatment decreases K_d by a factor of 10, the target-size analysis will indicate a size of 1.25 and thus could be interpreted as a decrease of the size of the active enzyme. It would be suggested that the treatment leads to the dissociation of a component from the active enzyme. Actually, the treatment does not induce the involvement of another component but only favours a tighter binding of R and E. Thus, in this case, a decrease of the apparent target size corresponds to an increase of the binding affinity.

The actual size of the active component, i.e. the RE complex, is equal to 2. The next simulation considers various sizes of R and E assuming that the sum of the sizes remains equal to 2. Fig. 4 shows that the apparent target size varies from 1.58 to 1.8, the minimum being obtained when $\mu_E = \mu_R = 1$.

All the above simulations were done with the assumption that free E does not express any enzymic activity, i.e. that the regulatory unit is absolutely required as an activator of the catalytic unit. This assumption is substantiated when $(k_{cat.})_E = 0$. In the next simulation the ratio $(k_{cat.})_{RE}/(k_{cat.})_E$ was de-

Fig. 3. Effect of changes in K_d , $[E_t]$ and $[R_t]$ on the apparent target size μ_{app} .

See the text for further details. The ratio $[R_t]/[E_t]$ is equal to 0.01 (curve 1), 0.1 (curve 1), 1 (curve 2), 10 (curve 3), 100 (curve 4).

creased. Fig. 5 shows that the apparent size decreases with this ratio. If the regulatory unit acts as a total inhibitor, i.e. $(k_{cat.})_{RE} = 0$, then the apparent target size is equal to 0.84. If the regulatory unit has no effect on the activity, i.e. $(k_{cat.})_{RE}/(k_{cat.})_E = 1$, then the apparent size is exactly equal to 1.

It has been repeatedly suggested in experimental studies (Kempner & Schlegel, 1979) that radiation energy would be transferred between coupled sub-units because of a sufficiently strong interaction between the molecules. In order to consider such a possibility, it is necessary to know how the molecules are distributed between free and bound forms at the beginning of the irradiation and whether these forms are interconvertible during the irradiation. There is no reason to believe that the equilibrium state of the sample at room temperature is preserved when the sample is cooled, dried or freeze-dried. Thus the fraction of bound molecules in the sample prepared for the irradiation procedure is virtually unknown. However, it can be assumed that, during the irradiation, the association of free molecules and/or the dissociation of the complex are slow because of the physical state of the sample, and thus possible kinetic effects due to the chemical reactions are neglected. Fig. 6 shows the influence of the distribution of free and bound molecules at the beginning of the irradiation on the apparent target size. If all the components are dissociated ($f_{Rb} = f_{Eb} = 0$), then the apparent size is equal to 1.58, which is, as expected, identical with the value obtained when the energy is not transferred. The apparent size increases with the fraction of bound molecules and reaches 3.16 when all the molecules are in the complex form. This value, which is twice the value obtained in the absence of energy transfer exceeds the size of the active complex by far. Thus,

Fig. 4. Effect of changes in the ratio between the relative sizes of E and R on the apparent target size μ_{app} .

See the text for further details.

Fig. 5. Effect of changes in the relative activities of RE complex and of free enzyme E on the apparent target size μ_{app} .

See the text for further details.

Fig. 6. Effect of the distribution of free and bound molecules at the beginning of the irradiation on the apparent target size μ_{app} .

f_{Rh} is the fraction of regulatory unit bound to the enzyme. It is assumed that the radiation energy is transferred between linked molecules. Thus both molecules of a complex are destroyed by a single hit. See the text for further details.

Discussion

The method of target-size analysis was first used on simple enzyme systems consisting of a population of one or several enzymes expressing the same function. Thus the enzyme inactivation consisted of one or a combination of exponential functions of the dose. The success of this approach had led to an extension of the methodology to the study of more complex systems, e.g. insulin binding to cell receptors (Harmon *et al.*, 1980), the calmodulin-dependent phosphodiesterases (Kincaid *et al.*, 1981) and the hormone-sensitive adenylate cyclase (Houslay *et al.*, 1977; Martin *et al.*, 1979; Schlegel *et al.*, 1979; Nielsen *et al.*, 1981). In these cases target-size analysis was essentially used to describe the structural organization of the components involved, but the validity of the interpretations has not been demonstrated so far. We have now approached this problem by assuming that an experimentally studied system is described by the regulatory-and-catalytic-units model characterized by a given set of parameter values and hypotheses (see the legend to Fig. 2). The question we asked was: if this system were experimentally studied by using the techniques of inactivation by irradiation, what would be the conclusions drawn from target-size analysis? Theoretical simulations of this model allow us to answer such a question (Figs. 2–6). The experimental results would lead to the conclusion that the enzymic activity is expressed by only one functional unit because of the apparent linearity of the decay curve (Fig. 2). This unit is characterized by a relative size of 1.57. On assuming that a certain treatment is known to activate the system and significantly decreases the apparent target size to 1.25, it would be concluded that the activation of the system by this treatment is concomitant with the dissociation of an inhibitory component from the enzyme. The calculated relative size of this component would be equal to 0.32. These interpretations are in fact compatible with the data provided that some constraints are fulfilled: the radiation energy is transferred between the catalytic unit and the partially inhibitory unit characterized respectively by relative sizes of 1.25 and 0.32, the concentration of catalytic unit is limiting, all the catalytic units are coupled to the inhibitory units in the control experiment and are dissociated when the considered treatment is applied. This description, based on a straightforward target-size analysis, would be embraced because it corresponds to the picture that intuition first predicts. However, these interpretations are really misleading, since they are in complete disagreement with the actual system. Indeed, these data were generated by a model involving a catalytic unit and an activator both characterized by a relative size of 1 (Fig. 2). The

provided that energy transfer occurs, target-size analysis may erroneously suggest the existence of a target bigger than any component involved in the system.

experimental treatment used was supposed to increase the affinity of the activator for the catalytic unit (see the Results section referring to Fig. 3). Moreover, it was assumed that energy transfer does not occur. It thus appears that target-size analysis cannot discriminate between these two interpretations, at least on the basis of experimental results that present some inaccuracy (5%) and for which the level of inactivation by irradiation does not exceed 95%. Moreover, the data cannot give any piece of information on the actual size of the active enzyme. Theoretically, this size is given by the slope at an infinitely high dose. However, the determination of this slope would require one to fit a straight line by linear regression through the data points corresponding to a nearly complete inactivation of the enzyme. This procedure does not seem to be experimentally realizable.

At this level of the study, the usefulness of target-size analysis applied to a complex equilibrium system can be questioned. Such a method would indeed be powerful if it could discriminate between working hypotheses. However, drastically different models can generate similar data. It appears that such a methodology increases the degree of freedom of the problem by introducing a further complexity in the system, e.g. the possible (not proved) existence of energy transfer, rather than decreasing this degree of freedom by rejecting some working hypotheses. Some examples taken in the literature exemplify this conclusion, as follows.

(1) For instance, activation of the system at lower radiation doses has been observed for insulin binding to cell receptors (Harmon *et al.*, 1980) and for the basal activity of calmodulin-dependent phosphodiesterases (Kincaid *et al.*, 1981). In the former case, the authors suggested the existence of a high-molecular-weight inhibitor of the binding activity. In contrast, in the latter case it was proposed that a mixed population of monomeric and dimeric phosphodiesterases, in which only the monomeric form is active in the basal condition, could account for the apparent activation by low radiation doses. This latter interpretation is in disagreement with theoretical simulations showing that a monomer-dimer equilibrium system can never be activated by irradiation (theoretical results not shown). This discrepancy is explained by the fact that Kincaid *et al.* (1981) assumed that this system did not reach a new monomer-dimer equilibrium after irradiation, i.e. during the assay of basal phosphodiesterase activity. This implicit assumption should be verified and thus the kinetics of monomer-dimer transformation should be characterized before one could interpret this target-size analysis.

(2) Using target-size analysis, Nielsen *et al.* (1981) proposed that the components of the adenylate cyclase system in turkey erythrocytes are

precoupled before activation by β -adrenergic agonists. These authors based their conclusion on the fact that the apparently linear decay curve of the ground state reveals an apparent target size equal to the sum of the sizes of the different components. In a previous study (Swillens & Dumont, 1981) we showed that a model where the components are uncoupled before activation can also generate such experimental results. In this more complex system it is evidently extremely difficult, if not impossible, to discriminate between different models.

In any case, the interpretation of target-size analysis is based on many assumptions, which should be explicitly stated. For instance, a working hypothesis on the mechanism of adenylate cyclase activation should be proposed, since the interpretation of the target-size analysis depends on whether the activation occurs through dissociation of the component or simply through a conformational change of the whole complex without dissociation. [Contrary to a previous assertion (Swillens & Dumont, 1981), Nielsen *et al.* (1981) did not assume any mechanisms of activation and proposed only that the system is precoupled before activation.]

(3) Using rat liver plasma membranes, Martin *et al.* (1979) showed how glucagon and [γ -imido]-GTP affect at different temperatures the apparent size of the adenylate cyclase system. These authors proposed a model that involved association and subsequent dissociation of different components of the system. The changes in the apparent size were thus attributed to a structural change of the system. Our present study has shown that it is practically impossible to discriminate between a structural change, i.e. the radiation inactivation is more efficient when the components are coupled, and a functional change, i.e. the apparent target size varies because of changes in the parameter values and not in the state of coupling during the irradiation.

The practical results of the present theoretical study can be summarized as follows. (a) Because of the inaccuracy of the experimental results obtained and of the current range of degrees of inactivation, the ' $\ln(v_D/v_0)$ -versus- D ' representation of the data generally appears to be linear. (b) When the data are generated by a complex system, the apparent target size can underestimate or overestimate by far the actual size of the active molecule. (c) The interpretation of target-size analysis is based on several assumptions, which should be explicitly stated. Unfortunately, among them are several unverifiable hypotheses, such as the existence of energy transfer between non-covalently linked subunits, the distribution of the components between free forms and bound forms at the beginning of the irradiation, and the equilibrium of the system during the irradiation and during the enzymic assay.

Conclusion

The aim of the present work was to demonstrate that target-size analysis applied to complex enzymic systems may lead to overinterpretations. From such a study, it is difficult to draw general positive conclusions, as they depend on the model chosen. Even on the basis of only one model, it is hard to obtain a complete analysis because of the high degree of freedom of the system. In the case of the regulatory-and-catalytic-units model, which is one of the simplest equilibrium systems, four independent parameters have to be considered, namely $K_d/[E_t]$, $[R_t]/[E_t]$, $(k_{cat})_{RE}/(k_{cat})_E$ and μ_R/μ_E , together with the assumption on the transfer of radiation energy between the molecules when they are coupled. If such an energy transfer occurs, further parameters describing the distribution of the molecules between the different free and bound forms must be introduced. It appears that the theoretical analysis of such a model can provide, at best, several examples of the behaviour of the system obtained for some particular sets of parameter values.

On the basis of such a theoretical study, it can be concluded that target-size analysis applied to complex systems cannot generally discriminate between models and cannot allow the correct determination of the target size. The only positive experimental result concerns the changes in the apparent target size due to some treatments. However, it should be kept in mind that, in this case, the number of possible explanations increases with the complexity of the system. Because of alternative hypotheses, which are currently unverifiable, the rejection of invalid explanations often remains impossible. One

can just hope that, for some particular complex systems, some alternative hypotheses can be rejected and thus the description of the system will be improved by target-size analysis. It will probably depend on the level of knowledge concerning the studied system acquired before the interpretation of radiation inactivation experiments.

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