Simulation of an inhibitory equilibrium system

Aberrant proteinic target sizes as obtained by radiation inactivation

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A model consisting of the reversible binding of an enzyme and a full inhibitor was theoretically studied. Radiation-inactivation experiments were simulated. Target-size analysis of the computer-generated data indicates that aberrant sizes much higher or much lower than the size of any component of the system may be obtained. Thus a target size larger than the sizes of the components of the system does not imply the existence of a multimeric functional unit of this size.

The inactivation of enzyme by irradiation has proved to be a useful method to determine the molecular mass of the functional enzymic unit *in situ* (Kempner & Schlegel, 1979). However, the simulation of the simple equilibrium system:

where E is the enzyme and R is a regulatory unit, indicated that target-size analysis may lead to misinterpretation, especially when the molecular organization of the different components of the system is unknown (Simon *et al.*, 1982). For instance, if R is an activator of the enzyme and if the radiation energy cannot be transferred between the non-covalently linked molecules, it can be shown that the apparent target size may take any value lying between the sizes of the components.

In the present paper I have pursued the study of the previously considered model assuming that R is a full inhibitor of the enzymic activity. The simulation of radiation inactivation and target-size analysis applied to this system suggests that aberrant target sizes much larger or much smaller than the size of any component may be obtained. Thus the present study gives a novel way to explain a too-large target size, which was interpreted up until now as resulting from the aggregation of several smaller components (Rodbell, 1984).

Model and methods

The theoretical methods used for the present study have been previously described by Simon *et al.* (1982). It is assumed that radiation energy is not significantly transferred from one component to

the other one within the non-covalently bound RE complex, and that the proteins are only submitted to the direct action of ionizing radiation, i.e. the experimental samples have to be freeze-dried or frozen. The former assumption is supported by recent results on glutamate dehydrogenase (Kempner & Miller, 1983). Thus the total concentrations of R and E are mono-exponential functions of the radiation dose, D:

$$[E]_D = [E]_0 e^{-\mu_E D} \quad (2)$$

$$[R]_D = [R]_0 e^{-\mu_R D} \quad (3)$$

where $[R]_0$ and $[E]_0$ are the total concentrations of E and R before irradiation.

Since R is a full inhibitor, the activity is proportional to free E concentration, which can be computed at each radiation dose by using eqns. (2) and (3) and the Mass Action Law derived from eqn. (1):

$$[E]_D = \frac{1}{2} \{ [E]_D - [R]_D - K_d + \sqrt{([E]_D - [R]_D - K_d)^2 + 4[E]_D K_d} \} \quad (4)$$

K_d is the dissociation equilibrium constant of the reaction. Different sets of parameter values have been considered. In each case, the decay curve, $\ln(v_D/v_0)$ versus D was determined for D varying between 0 and the dose leading to a 5% residual activity. Furthermore, 11 points were generated within this range assuming a random error on the activity characterized by a 5% coefficient of variation [see Simon *et al.* (1982) for further details]. This procedure allows a visual appraisal of the curvature of the decay curve as it would have been experimentally obtained.

According to Kepner & Macey (1968), we assume that the slope, μ , of the decay curve is linked to the molecular mass by the empirical equation:

$$\text{Molecular mass} = 6.4 \times 10^8 \times \mu \quad (5)$$

where the molecular mass and μ are expressed in kDa and Mrad⁻¹ respectively.

Results

Setting the value of $[R]_0$ and $[E]_0$ to 1 (arbitrary concentration unit), a value of 10^{-3} has been chosen for K_d , allowing the expression of 3.11% of the total activity. If the inhibitor size is significantly lower than the enzyme size (30 kDa and 100 kDa respectively), a very large apparent target size is obtained at low radiation doses (Fig. 1a). Indeed, the slope at the origin corresponds to a size of 1160 kDa, i.e. more than ten times larger than the size of the functional unit, E. Even at the dose leading to a 5% residual activity, the slope still leads to an overestimated value of 185 kDa. Much higher doses should be delivered in order to allow a better estimate of the actual size of E. Fig. 1(a) clearly displays a non-linear decay curve in spite of the 5% random error. In order to see whether this aberrant size is always associated with an apparent curvature of the decay curve in the considered domain of residual activities, the relative concentrations of E and R were modified. Fig. 1(b) shows that, with a value of 0.89 for $[R]_0$, the curvature is much less pronounced and might be masked by a 5% random error. However, the apparent size remains much larger than the actual size of E, since it varies from 1060 kDa at mid-curve to 500 kDa at the highest dose. Fitting of the data points by linear regression leads to an apparent target size of 840 kDa. It has to be stressed that the shape of the curve, and thus the interpretation of the results, are sensitive to a slight modification (11%) of the relative concentrations of the components.

The existence of an aberrant large size as given by target-size analysis is partly due to a small size of the inhibitor as compared with the size of the enzyme (see Fig. 1a). In the next simulation, equal sizes of 100 kDa have been considered for E and R. Fig. 1(c) shows that, in this case, the decay curve appears to be linear, even in the absence of random error. Moreover, the slope corresponds to a size of 53 kDa, which underestimates the actual size of the functional unit. Theoretical considerations indicate that such a linear decay curve exhibits the minimal obtainable slope when, at any radiation dose, D , K_d is much lower than $[E]_D$ and $[R]_D$, and much higher than $[R]_D - [E]_D$, which must be non-negative. In this case the corresponding size is equal to half the size of E. In order to fulfil these

severe constraints, it appears that the sizes of E and R must be quite similar. Therefore it can be anticipated that the shape and slope of the decay curve are highly sensitive to a change of the relative sizes of R and E. If the size of E is slightly smaller than the size of R (90 kDa and 100 kDa respectively), the decay curve exhibits a maximum (Fig. 1d). This increase of activity at low doses results from a preferential degradation of the inhibitor, owing to its larger size. This phenomenon has been previously detected for insulin binding to cell receptors (Harmon *et al.*, 1980). It has been noted that the slope of the linear part of the curve displayed at higher doses corresponds to a target size of 72 kDa, which underestimates the actual size of the functional unit. Fig. 1(e) shows the results obtained in the opposite situation, i.e. the sizes of R and E are equal to 90 kDa and 100 kDa respectively. The decay curve becomes qualitatively similar to the one presented in Fig. 1(a), i.e. apparent curvature and aberrant high target size (209 kDa) obtained at low dose. However, the size determined at higher doses is equal to 40.5 kDa, which is quite lower than the size of E. Again, the actual size will be approached at much higher radiation doses, leading to much lower residual activities. For instance, a size of 98 kDa would be measured at a dose of 80 Mrad, leading to a 0.01% residual activity.

All the presented results have been obtained with a K_d equal to one-thousandth of the initial concentration of E. If the affinity was lower, the degradation of the inhibitor would affect the radiation inactivation of the enzyme to a lesser extent. Thus the curvature would be less pronounced and the slope would become compatible with the size of the enzyme (results not shown). By contrast, an increase of affinity would amplify the general characteristics displayed in the Figures (results not shown).

Discussion and conclusion

Enzyme inactivation by irradiation was recognized as a unique tool to estimate the size of a functional molecule in its natural environment, e.g. the cell membrane. Because the biological function of key molecules is generally regulated through the interaction with regulatory proteins, the radiation-inactivation methodology is currently applied to complex systems. In some cases, the complexity of the system might be underestimated or simply ignored by the experimenter. Consequently this methodology might lead to a bad estimate of the target size and possibly to over-interpretations, even if the decay curve displays apparent linearity (Simon *et al.*, 1982). On the other hand, if the complexity of the system is not

Fig. 1. Target-size analysis of computer-generated data. The simulation of the model defined by eqn. (1) (see the Model and methods section) accounts for different parameter values as follows: $K_d = 10^{-3}$;

ignored, it seems hard to give a correct interpretation of the target-analysis results unless the actual description of the system was previously proposed on the basis of other experimental data. In our opinion, target-size analysis should serve essentially as a means to validate or invalidate working hypotheses and not to propose new interpretations. It is the purpose of the present paper to illustrate this point by simulating a complex system consisting of only two components.

Non-linear decay curve and/or aberrant target size are usually indicative of the complexity of the system. Previous simulations have shown that data generated by the system described by eqn. (1) were generally not indicative of any complexity when R is an activator (Simon *et al.*, 1982). On the contrary, the present study demonstrates that the existence of an inhibitor of the enzyme activity may lead to non-linear decay curves and aberrant target sizes. The ultimate reason for these results is the competition between the inactivation process of enzyme destruction and the activation process of inhibitor destruction. Depending on the relative concentrations and sizes of these two components, the decay curve may display very different shapes and slopes. Only the slope obtained at an infinitely high dose is independent of the parameters of the system, simply because the enzyme concentration becomes much lower than K_d . Thus this slope corresponds to the size of the functional unit, E. The question of whether this slope can be approached at the highest doses deposited in the system is generally unanswerable unless the model and its parameters are known.

Hormone-responsive adenylate cyclase is a complex system in which several components are involved. The role of these components and their molecular organization inside the cell membrane have been described (Smigel *et al.*, 1984). Briefly, the hormone-receptor complex interacts with a 35kDa protein of a heterodimer leading to the dissociation of the other moiety (45kDa). The dissociated 45kDa protein is then capable of activating adenylate cyclase. In other words, the stimulation of adenylate cyclase activity results from a cascade of three main events: the transient interaction of the hormone-receptor complex with the 35kDa protein, the dissociation of the 45kDa transducer from the 35kDa inhibitor, and the as-

$[E]_0 = 1$; $[R]_0 = 1$, except in (b), where $[R]_0 = 0.89$; the sizes of E and R are respectively equal to 100kDa and 30kDa (a), 100kDa and 30kDa (b), 100kDa and 100kDa (c), 90kDa and 100kDa (d), 100kDa and 90kDa (e). The continuous lines describes the theoretical decay curves, whereas the points take a 5% random error into account.

sociation of the active transducer with adenylate cyclase. It is clear that such a system, which is much more complex than the one analysed in the present study, may produce by simulation any sort of decay curve. It has been reported that, in rat liver and adipocytes, the radiation inactivation of the glucagon-responsive adenylate cyclase is characterized, under some experimental conditions, by a non-linear decay curve and by an aberrant large size of 1300 kDa (Schlegel *et al.*, 1979). Up until very recently, it has been repeatedly claimed that this high apparent-size value is indicative of the existence of a large multimeric form of the components (Rodbell, 1984). The proposed theoretical study suggests that the complexity of the minimal model describing the adenylate cyclase system is sufficient to explain the experimental data. The presence of a 35 kDa protein capable of inhibiting the larger transducer protein (45 kDa) is in fact a prerequisite to obtain an aberrant high target size. Invoking the aggregation of the regulatory components thus introduces a further unnecessary complexity in the system.

The main contribution of our theoretical study is to show that apparent size of the so-called functional unit deduced from target-size analysis may be drastically different from the size of any molecule or molecular complex that actually exists in the system. In fact, the slope of the decay curve

reflects the sensitivity of the biological function to irradiation. That this sensitivity depends on the size of the molecules involved in the expression of the function is evident. However, one should be aware of the possible role of other parameters like equilibrium constants and component concentrations in affecting this sensitivity.

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