Radiation-Target Molecular Weights of Urease and of L-Glutamate Dehydrogenase, and their Relevance to the Size of the Functional Subunits

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(Received 12 November 1970)

Activities of the oligomeric enzymes urease and L-glutamate dehydrogenase were measured after exposure as dry preparations to various doses of electron radiation. Inactivation curves were exponential. 'Target sizes' deduced from these were small compared with the molecular weights of the whole enzyme molecules, but accorded well with independent estimates of the sizes of functional subunits. It was concluded that, when these were in associated form, there could be no transfer of absorbed energy between subunits.

A radiation 'target volume' is defined as that volume within which a single quantum of absorbed energy results in loss of biological activity. On the assumption that the average 'energy-loss event' imparts 60-110 eV, target volumes for both proteins (Hutchinson & Pollard, 1961) and the nucleic acid cores of viruses (Alper & Haig, 1968) correspond reasonably well with independent determinations. With some proteins, however, 'target' molecular weights have been found to be considerably smaller than those determined by biochemical methods, and a likely explanation is that until recently those methods have given estimates of the molecular weights of oligomers rather than of functional units.

The object of this investigation was to determine target molecular weights for urease and L-glutamate dehydrogenase. These were chosen because their physical substructure and the sizes of the active subunits are still subjects of debate. The enzymes were irradiated in the dry state to avoid 'indirect effects', i.e. contributions to inactivation by radiolysis products of water.

MATERIALS AND METHODS

Preparation for irradiation

Urease. Jack-bean-meal tablets (25 mg of protein/tablet) were supplied by Dunning Products (Hynson Westcott and Dunning Inc., Baltimore, Md., U.S.A.), and purified crystalline urease (2000 units/g of protein) by Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. One unit of urease activity is defined as that amount of enzyme which will produce 1 mg of ammonia nitrogen from urea in 5 min at pH 7.0 at 30°C. Since these were dry materials, no preparation was required other than the dispensing of weighed amounts into glass ampoules.

L-Glutamate dehydrogenase. This was supplied by Sigma in suspension containing strong electrolytes. Portions

(0.5 ml) of the suspension were freeze-dried in glass ampoules for irradiation; but it was necessary to check that the other solutes, added to protect against loss of enzyme activity, did not protect the enzyme against, or sensitize it to, the inactivating action of radiation. The solution was therefore dialysed overnight against 0.05 M-potassium phosphate buffer (concentration 1 mg/ml) at pH 7.6 at 4°C. The dialysed solution was diluted to 0.2 mg/ml to give optimum concentration of protein for freeze-drying, for which 0.5 ml samples were dispensed in glass ampoules, frozen, and evacuated under 0.02 mmHg for 4-6 h. The powder so obtained withstood at least 24 h storage at 4°C without loss of activity. Assays were always made within a day after irradiation.

Irradiation and dosimetry

The glass ampoules were exposed to electron beams either from the Medical Research Council linear accelerator or from a 2 MeV van de Graaff generator. They were open during irradiation to avoid protection by anoxia, and cold air was blown over them to assist in the dissipation of heat. Doses were monitored during irradiation by suitably located air-ionization chambers, the monitors being calibrated by simultaneous exposure of clear Perspex (Boag, Dolphin & Rotblat, 1958), or 300 MSC cellophane of 0.001 in thickness impregnated with blue dye (product of Du Pont, U.S.A.). The absorption of radiation dose caused bleaching of the dye (Henley & Richman, 1956). Dosimetry by blue cellophane and Perspex were related to calorimetric measurements made by Bewley (1969). Errors in dosimetry were within $\pm 5\%$ and $\pm 3\%$ respectively with the blue cellophane and the Perspex. The glass walls of the ampoules were thick enough to give full 'build-up' of dose rate.

Enzyme assays

Urease. The method used was basically that of Van Slyke & Archibald (1944), which depends on the measurement of the amount of $(\text{NH}_4)_2\text{CO}_3$ evolved in a given time,

so that rate of change of pH value is a measure of enzyme activity. Measurements were made by recording the time taken for a solution of enzyme and substrate in phosphate buffer to reach the pH value of a solution of enzyme in buffer, with no substrate. The solutions used were as follows: reaction tube, 3 g of urea in 100 ml of water containing 10 ml of 1.0 M-NaH₂PO₄ and 10 ml of 1.0 M-K₂HPO₄, pH 6.7; control tube, 1 ml of 1.0 M-NaH₂PO₄ and 7 ml of 1.0 M-K₂HPO₄ in 80 ml of water, pH 7.7. At zero time, 0.5 ml of the urease solution under test (concentration 0.10–0.40%, w/v) was added to each of two tubes containing 5 ml of reaction and of control solutions. The time taken for the pH values to become equal was recorded. Observations were limited to a change in pH value of only 1 unit, to avoid the possible effect of grosser pH changes on reaction rate. Within this range, the pH rose linearly with time, and the slope of the line was a convenient measure of reaction rate.

L-Glutamate dehydrogenase. The assay technique was based on that of Adelstein & Vallee (1958) and Strecker (1955). The overall reaction catalysed by glutamate dehydrogenase can be written:

and the production of NADH is readily followed spectrophotometrically at 340 nm.

Activities of irradiated and non-irradiated samples of the enzyme were compared by mixing the following in quartz cuvettes: 2.5 ml of 0.05 M-potassium phosphate buffer, pH 7.6; 0.1 ml of 0.01 M-NAD⁺; 0.2 ml of potassium glutamate solution; 0.2 ml of L-glutamate dehydrogenase solution (0.002% protein in 0.05 M-potassium phosphate buffer, pH 7.6). The cuvettes were placed in a Unicam sample holder maintained at 25 ± 1°C, and their *E*₃₄₀ values recorded every 30 s for 5 min. Change in *E*₃₄₀ per unit time was taken as a measure of reaction rate.

With both enzymes, the residual activity of an irradiated sample was defined by the ratio of the reaction rate to that of the control. This procedure was justified by checking that the decreased reaction rates with irradiated samples could be matched by using unirradiated samples of correspondingly lower concentration.

RESULTS

The results are shown in Figs. 1 and 2, as semi-logarithmic plots of residual activity against radiation dose. The inactivation curves are the best lines fitted to the sets of points by the method of least squares. Within experimental error, inactivation curves were exponential, and were not affected by the presence of impurities, since they were the same for unpurified and purified urease, and were also the same for glutamate dehydrogenase, whether or not the suspensions were dialysed before being dried. 'Target sizes' are commonly calculated from the dose *D*₀ required to give an average of one inactivating event per active unit, i.e. the dose that will leave a residual activity of *e*⁻¹ (36.8%) of the non-irradiated control. Values of *D*₀ were respectively 7.86 and 2.59 Mrd (megarads) for urease and glut-

Fig. 1. Effect of electron irradiation on urease activity. Radiation dose is plotted against activity recovered for unpurified enzyme tablets (●) and for pure crystalline enzyme (○). Activity loss was related to the time taken for irradiated enzyme solution (irradiated dry) to reach pH value of control solution.

Fig. 2. Effect of electron irradiation on the activity of L-glutamate dehydrogenase. The activity of the irradiated enzyme was measured, after the oxidation reaction of L-glutamate dehydrogenase in the presence of coenzyme NAD. The rate of reduction of NAD⁺ to NADH was measured spectrophotometrically by recording *E*₃₄₀ at the initial stage of the reaction. ●, Activity versus dose for dried commercial preparation; ○, activity versus dose for dialysed pure enzyme.

amate dehydrogenase, and, within experimental error, both inactivation curves were exponential. According to the theory of Lea (1946), the 'target' molecular weights corresponding to these values of *D*₀ are respectively 9 × 10⁴ and 3 × 10⁵.

DISCUSSION

The theory for calculating 'target molecular weights' from radiation-inactivation data was developed and refined by Lea (1946), who took into account the clustering of ion pairs and the tendency for ionizing events to occur in tracks rather than spatially at random. The Lea (1946) calculations, used here to estimate the molecular weights of the active units of urease and glutamate dehydrogenase, are based on the assumption that the size of the average 'energy-loss event' is 100eV (a cluster of three events of 33eV each). Rauth & Simpson (1964) measured the distribution of energy lost per ionizing event in aluminium and Formvar ($C_5H_8O_2$ polyvinyl formal) and found an average value of 60eV for 20 KeV electrons. Marshall, Holt & Gibson (1970) reported that on average 46eV/event was deposited by 1MeV electrons in dry ribonuclease, and that the value increased slowly with energy. Since the estimate of target size by Lea's (1946) methods is positively correlated with the event size, estimates of molecular weight based

on Lea's (1946) calculations would be too high by any factor by which he overestimated the energy of the average deposition event.

In these laboratories, however, various materials ranging in molecular weight from 5×10^4 (Fab' fragment of globulin; T. Alper, M. Sterne & H. Warrack, unpublished work) to 6×10^7 (Alper & Haig, 1968) have been irradiated by 8MeV electrons under the same conditions, i.e. freeze-dried and with oxygen present, and reasonably good agreement between radiobiologically and independently determined molecular weights has been obtained by using the Lea (1946) theory. It has therefore also been used in the present paper. Since other estimates of the average energy absorbed from an electron beam per ionizing event have been lower than Lea's (1946), the estimates of target molecular weight for glutamate dehydrogenase and urease reported here may be regarded as maximal. In point of fact, as shown below, the estimates based on Lea's (1946) theory agree reasonably well with some estimates of the size of functional subunits of these enzymes.

Table 1. *Various estimates of molecular weights of urease*

Mol.wt.	Reference	Methods and remarks
(a) Oligomeric state		
4.83×10^5	Sumner, Gralen & Eriksson-Quensel (1938)	Sedimentation velocity and diffusion measurements
2.4×10^5 (approx.)	Sehgal & Tanis (1965)	Sucrose-density-gradient centrifugation
3.7×10^5	Reithel & Robbins (1967)	High-speed sedimentation equilibrium method, in dilute solution
2.6×10^5	Tanis & Naylor (1968)	Sucrose-density-gradient analysis
2.4×10^5	Gorin, Chin & Wang (1968)	Dissociation in acetate buffer; retention of enzymic activity
(b) Subunit		
9.2×10^4	Setlow (1952)	Inactivation by deuterons
8.3×10^4	Reithel, Robbins & Gorin (1964)	Ultracentrifugation with guanidine hydrochloride
7.5×10^4	Bailey & Boulter (1969)	Polyacrylamide-gel electrophoresis

Table 2. *Various estimates of molecular weights of glutamate dehydrogenase*

Mol.wt.	Reference	Methods and remarks
10^6	Olson & Anfinsen (1952)	Sedimentation and diffusion measurements
4.5×10^5 – 1.25×10^6	Kubo, Iwatsubo, Watari & Soyama (1959)	Light-scattering
4.3×10^4	Jirgenssen (1961)	Sedimentation
8×10^4 – 2×10^6	Fisher, Cross & McGregor (1962)	Light-scattering
1.1×10^5	Horne & Greville (1963)	Electron-microscopic examination (suggested subunit value)
1.3×10^6 – 1.4×10^6	Frieden (1964)	Light-scattering; suggested maximum mol.wt.; molecule composed of four active subunits
2.5×10^5 – 4.0×10^5	Magar (1965)	Sedimentation and viscosity analysis; mol.wt. for active unit
3.13×10^5	Eisenberg & Tomkins (1968)	Light-scattering mol.wt. for active unit, composed of six non-active polypeptides each of mol.wt. 5.3×10^4

Table 3. *Subunit sizes for some enzymes estimated from radiation studies*

Enzyme	Total mol.wt.	Subunit mol.wt. inferred from target volume	Reference
Yeast alcohol dehydrogenase	1.5×10^5	3.7×10^4	Hutchinson, Preston & Vogel (1957)
Yeast alcohol dehydrogenase	1.5×10^5	3.6×10^4	Kägi & Vallee (1960)
Xanthine oxidase	3×10^5	7.5×10^4	Fluke & Hochachka (1965)
Lactate dehydrogenase	1.4×10^5	3×10^4 – 4.5×10^4	Fluke & Hochachka (1965)

Tables 1, 2 and 3 summarize reports from the literature of the aggregate molecular weights of the two enzymes used and of the molecular weights of the functional subunits.

In summary, the results reported here confirm that when oligomeric enzymes are irradiated in the dry state, the target size is a reasonably good measure of the molecular weight of the active subunit rather than of the whole molecule. The corollary is that although the deposition of energy by ionizing radiation anywhere within the subunit will cause loss of activity, energy will not be transferred to the associated subunits; if it were, the target size would be larger. It is also worth commenting on the exponential nature of the inactivation curves, which demonstrate that each subunit acts independently of the others as far as this analysis is concerned. If an oligomer consisted of n subunits, each having activity, but with the activities remaining associated, the inactivation curve would not be exponential, but would demonstrate a 'hit number' of n .

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