Radiation inactivation analysis of kidney microvillar peptidases

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Five membrane peptidases were studied by radiation inactivation analysis of pig kidney microvillar membranes. One heterodimeric enzyme, γ-glutamyl transferase, presented a target size corresponding to the dimeric M_r. The other enzymes are known to be homodimers. Three of these, aminopeptidase A, aminopeptidase N and dipeptidyl peptidase IV, gave results clearly indicating the monomer to be the target and, hence, in this group the association of the subunits was not essential for activity. The target size for endopeptidase-24.11 was intermediate between those for monomer and dimer and its functional state was not resolved by the experiments.

Brush border membrane *Membrane peptidase* *Microvillus* *Radiation inactivation analysis*

1. INTRODUCTION

All of the brush border peptidases that have been purified from pig kidney or intestine have been found to be dimeric in structure and this characteristic was not affected by the agent (proteinase or detergent) used to release the enzymes from the membrane (review [1]). The association of the two subunits of these enzymes does not therefore depend upon the hydrophobic anchor or transmembrane domains that are lost by the proteinase treatment. The assumption is usually made that the oligomeric state of the isolated protein reflects that existing in the membrane. This assumption has been supported by electron microscopy of a few enzymes that have been reconstituted into liposomes, e.g. aminopeptidase N (EC 3.4.11.2) [2], endopeptidase-24.11 (EC 3.4.24.11) [3] and dipeptidyl peptidase IV (EC 3.4.14.5) [4]. However, we know very little about the functional significance of the oligomeric state for these enzymes. One of them, γ-glutamyl transferase (EC 2.3.2.2), is a heterodimer, requiring both subunits for activity [5]. The other microvillar peptidases that have been studied are homodimers and for these enzymes no such information is available. Radiation inactivation analysis provides an independent approach to the question. The dose-dependent inactivation of an enzyme is related to the target size it offers to the high-energy beam (review [6]). If each subunit functions independently of the other the target size should approximate to the subunit size; if association is a prerequisite for activity the target should relate to the dimeric M_r. Here, we have tried to answer this question in regard to five peptidases in pig kidney microvilli (four homodimeric and one heterodimeric).

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2. EXPERIMENTAL

2.1. *Preparation of microvillar membranes*

Microvillar membranes were prepared from fresh pig or rabbit kidneys as described in [7] and resuspended in 1 mM K_2CO_3/HCl, pH 7.4, at 10 mg protein/ml.

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2.2. *Irradiation of membranes*

Aliquots containing 2 mg microvillar protein were freeze-dried in 1 ml plastic auto-analyser cups (base area 0.8 cm^2). Capped samples were irradiated in triplicate at room temperature with electrons from a Van der Graaf accelerator at the Radiation Research Centre, Cookridge Hospital, Leeds, operating at 2.9 MeV to give a dose rate of 20 krad/s. Electron flux was monitored continuously by measuring the current induced in a brass plate (230 cm^2) located beneath the samples and the dose rate integrated with respect to time to determine the exact dose delivered. The intensity of the electron beam has a Gaussian distribution over the target area where $\sigma = 6.5$ cm. In the circle (1.4 cm radius) in which the sample cups were irradiated the predicted radiation dose varied by only 2% from the centre to the circumference. No significant rise in temperature of the samples was detected over the dose range used. Non-irradiated controls were handled in an identical way. After irradiation samples were rehydrated in 1 ml of 10 mM Tris-HCl, pH 7.4, 2% (w/v) Triton X-100, at 20°C for 1 h, and any insoluble material removed by centrifugation. In one experiment the recovery of freeze-dried protein from the cups was monitored by including ^{125}I-labelled protein A with the membranes.

2.3. *Enzyme assays*

Aminopeptidase N, aminopeptidase A (EC 3.4.11.7), γ-glutamyl transferase, dipeptidyl peptidase IV and endopeptidase-24.11 were assayed in duplicate as described [8]. Protein was determined by the Lowry method.

2.4. *Calculation of target size*

Apparent molecular target sizes were calculated from the empirically established relationship [9] that $M_r = 6.4 \times 10^5/D_{37}$, where D_{37} is the radiation dose expressed in Mrad required to reduce the specific activity of the enzyme to 37% (i.e. $1/e$) of its original value.

3. RESULTS

3.1. *Sample preparation and recovery of protein from the cups*

In these experiments the freeze-dried samples of membranes were irradiated in air. A preliminary experiment was performed to determine if samples irradiated in this way yielded different results from those presented, more conventionally, in sealed evacuated glass vials. In this experiment triplicate samples were irradiated at four dose levels: 0, 2, 5 and 10 Mrad. The results for two enzymes, aminopeptidase N and endopeptidase-24.11, are shown in table 1. Since only 4 points were used to compute the first-order decay, the D_{37} values are less reliable than those shown in fig.1 where 15 points were used. However, it is apparent that the coefficient of determination (r^2) is slightly higher for the enzymes irradiated in air. More importantly, the variance among the data from the samples in cups was significantly lower than those in vials. We conclude that in spite of the possible

Table 1

Comparison of irradiation of samples in cups in air with those in evacuated glass vials

Enzyme	Vials in vacuo		Cups, in air		F statistic (V_{vials}/V_{cups})
	D_{37}	r^2	D_{37}	r^2	
Endopeptidase-24.11	4.93	0.912	4.58	0.983	79 ($p < 0.01$)
Aminopeptidase N	3.61	0.915	4.63	0.974	17 ($p < 0.05$)

See section 2 for details. The data are from triplicate samples irradiated at 0, 2, 5, 10 Mrad, with assays of two enzymes in duplicate. The variances (V) of the means for vials and cups were computed and the means of these were used to generate the F statistic

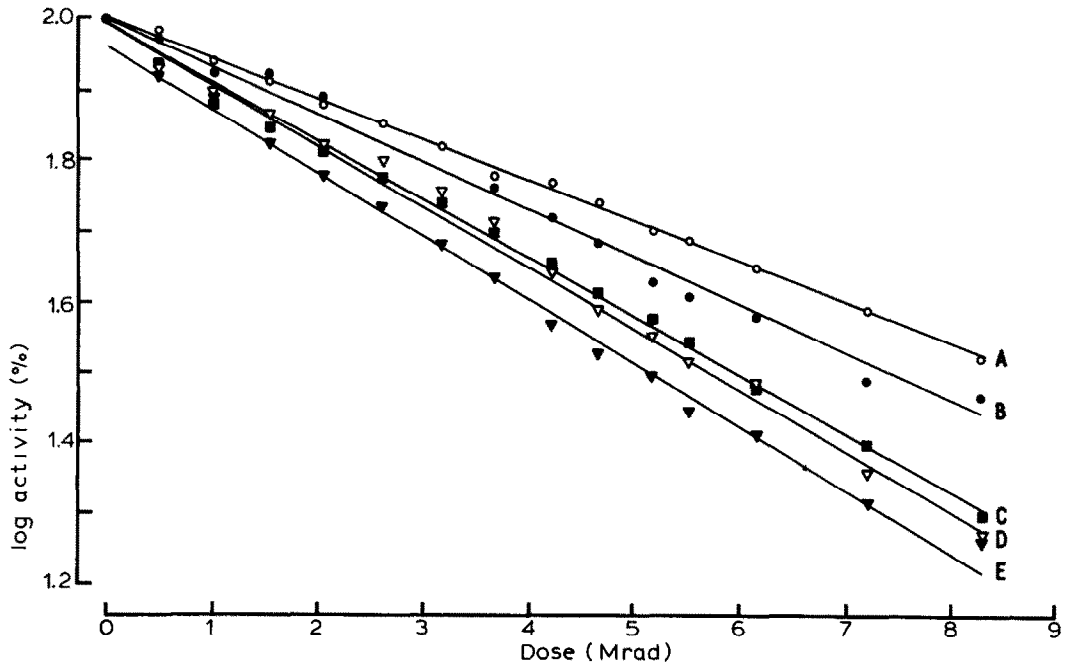

Fig.1. Radiation inactivation decay curves of kidney microvillar enzymes. See section 2 for details. Each point is the mean of two irradiation experiments. Enzymes: A (○) γ-glutamyl transferase; B (●) dipeptidyl peptidase IV; C (■) aminopeptidase A; D (▽) endopeptidase-24.11; E (▼) aminopeptidase N. D_{37} values and coefficients of determination are shown in table 2.

Table 2

Radiation inactivation analysis of pig and rabbit kidney microvillar proteins

Enzyme	Membrane activity				Purified enzyme		
	D_{37} dose (Mrad)	r^2	Target ($M_r \times 10^{-3}$)	No. of experiments	Subunit ($M_r \times 10^{-3}$)	Oligomeric state	Reference
Endopeptidase-24.11							
(pig)	4.96	0.990	129	3	89	2	[8]
(rabbit)	4.78	0.974	134	1	93	1	[10]
Aminopeptidase N							
(pig)	4.57	0.991	140	3	160	2	[11]
(rabbit)	4.64	0.983	138	1	136	1	[12]
Aminopeptidase A	5.20	0.996	123	2	170	2	[13,14]
Dipeptidyl peptidase IV	6.40	0.990	100	2	130	2	[15]
γ-Glutamyl transferase	7.53	0.999	85	2	54+27 (hetero-dimer)	2	[19]

The D_{37} dose is the mean of 3, 6 or 9 determinations (assayed in duplicate), as is the coefficient of determination (r^2). The values given for the target M_r ($\times 10^{-3}$) are means where more than one irradiation was performed. Information from SDS-polyacrylamide gel electrophoresis and other techniques has been used to indicate what is known of subunit size and oligomeric state of these enzymes when purified in their detergent forms (except for rabbit kidney endopeptidase-24.11 and aminopeptidase N, which were the proteinase forms). The values for γ-glutamyl transferase refer to rat kidney; in pig they are 59+33 (Hughey, R., personal communication)

theoretical disadvantages of irradiation in air, the use of cups was not only simpler, but gave more precise estimates of inactivation.

Recovery of enzyme activity after freeze-drying in cups was satisfactory for the five enzymes studied, the change in specific activity (units/mg protein) being −1.6% (range 5.8 to −4.8%). Recovery of radioactivity in the experiment in which ^{125}I-protein A was added before irradiation was equally efficient, all values were in the range 92–98% of the added radioactivity.

3.2. *Inactivation analyses*

Linear, first-order inactivation plots were obtained for each of the five enzymes studied (fig.1), each plot being the mean of two irradiation experiments. In another irradiation experiment, membranes prepared from rabbit kidneys were compared with those from pig. Linear regressions for endopeptidase-24.11 and aminopeptidase N were computed. Table 2 shows the D_{37} doses, r^2 values and the independent estimates from these experiments, of the target M_r values. Information on the apparent subunit M_r values of the detergent forms of the enzymes derived from SDS-polyacrylamide gel electrophoresis and the oligomeric state are included for comparison with the experimental data.

4. DISCUSSION

The enzymes studied are all known to be dimeric when purified from pig kidney, but endopeptidase-24.11 and aminopeptidase N are monomeric when isolated from rabbit. They are glycoproteins and a relatively small part of their mass is embedded in the lipid bilayer of the membrane. The target M_r values in table 2 are generally lower than the subunit M_r values for the purified peptidases, with one exception – endopeptidase-24.11. Here the values were consistently 45% higher and there was no difference between the pig enzyme (known to be dimeric) and that from rabbit (known to be monomeric). Since all these ectoenzymes are glycoproteins, one may enquire whether the carbohydrate moiety contributed to the target size. This point has been addressed in respect of yeast invertase [16], where it was found that removal of carbohydrate by treatment with endoglycosidase H did not alter the target size. We have some information on the deglycosylated M_r values for four of the peptidases obtained by endoglycosidase-H treatment of their labelled precursor forms [13]. These M_r values were: endopeptidase-24.11, 77000 (confirmed by chemical deglycosylation [17]); aminopeptidase N, 115000; aminopeptidase A, 120000; and dipeptidyl peptidase IV, 89000. The target M_r values for the three exopeptidases were therefore intermediate between those for the glycosylated and deglycosylated forms. Endopeptidase-24.11 gave values that were higher than the glycosylated subunit M_r. In this instance it is difficult to say whether the target is likely to be the dimer or the monomer, but since the rabbit endopeptidase is monomeric dimerization does not appear to be mandatory for activity. A similar conclusion can be made in respect of rabbit aminopeptidase N.

The three exopeptidases, all homodimers in pig, clearly present a target that approximates to the subunit size. γ-Glutamyl transferase, the only heterodimer in the group, gave a target very close to the dimeric M_r, including the contribution of carbohydrate chains. Our value agrees well with that observed by irradiation of rat kidney microvillar membranes [18].

The question posed at the outset concerning the relationship of dimeric structure to enzyme activity has been satisfactorily answered for four of the enzymes. We confirm that γ-glutamyl transferase activity depends on the association of large and small subunits. However, the three exopeptidases, aminopeptidases N, A and dipeptidyl peptidase IV offer target sizes that relate to the M_r of the deglycosylated subunit. For endopeptidase-24.11 the question was not clearly resolved by this approach.

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