Functional lysosomal hydrolase size as determined by radiation inactivation analysis

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Electron inactivation analysis with 16 MeV electrons was used to determine the functional target size of a number of commonly studied lysosomal hydrolases. Observed values ranged from a low of $62\,000 \pm 4\,000$ Da for β -galactosidase to a high of $200\,000 \pm 17\,500$ Da (mouse β -glucuronidase). One group of lysosomal hydrolases (*N*-acetyl- β -glucosaminidase, *N*-acetyl- β -galactosaminidase, α -galactosidase, β -mannosidase, β -glucosidase, arylsulphatase A and sphingomyelinase) had target sizes in the range 100 000–120 000 Da, whereas α -glucosidase and α -fucosidase exist as complex multimers in the 150 000–160 000 Da range. Analysis of freeze-dried cell material showed little evidence of species (mouse versus human) variation in the functional size of most lysosomal hydrolases with the exception of β -glucuronidase. Our findings suggest the potential usefulness of lysosomal hydrolases as endogenous marker enzymes in studies where the target size of proteins of unknown molecular mass is to be determined.

Radiation inactivation analysis is now well-established as a technique which can be used to estimate molecular masses of both soluble and membrane-bound peptides, usually enzymes or receptors (Kepner & Macey, 1968; Levinson & Ellory, 1974; Houslay *et al.*, 1977; Kempner & Miller, 1983). The equation normally used to relate molecular mass to inactivation dose has been justified both theoretically (Lea, 1955) and empirically (Kepner & Macey, 1968) for data that fit a simple mono-exponential relationship, although analysis of more-complex results in terms of subunit interactions or hormone–receptor complexes has been questioned (Simon *et al.*, 1982; Verkman *et al.*, 1984).

The present work applies radiation inactivation analysis to various lysosomal hydrolases in cultured cells, principally human skin fibroblasts and mouse neuroblastoma cells, and has three separate aims. One obvious and important reason for analysing certain hydrolases that have previously been well characterized is to act as internal standards in future irradiation work. If the irradiated samples give reproducible values that correspond to molecular sizes determined previously by other techniques, then this can be used routinely as an internal control to validate the method (McLawn *et al.*, 1983*a,b*). In the present work, such a correspondence was clearly achieved

with the enzymes *N*-acetyl- β -hexosaminidase and β -galactosidase.

More interestingly, radiation inactivation often gives a target size corresponding to a fraction, or multiple, of the molecular mass determined by other methods (Dawson, 1978), thus providing information about oligomerization or subunit structure of the enzyme. Lysosomal hydrolases are a very suitable group of enzymes for this type of study. Finally, the method can be applied to lysosomal enzymes, such as β -mannosidase, about which there is no information currently available with regard to molecular mass. Such results have obvious value in future purification and structural studies.

In the present work we have examined 12 separate enzymes, which provide examples of all three categories outlined above. Taken overall, the results underline the usefulness of radiation inactivation analysis in studying peptide molecular sizes, and provide good evidence for the validity of this method.

Materials and methods

Cell culture conditions

Human skin fibroblasts were cultured from frozen stocks maintained at the University of

Chicago as described previously (Dawson *et al.*, 1972). Mouse neuroblastoma $\times$ rat glioma hybrid cell line NG108-15 was obtained from Dr. W. A. Klee (National Institutes of Health, Bethesda, MD, U.S.A.) and NCB-20 cells were obtained from Dr. J. Minna (Veterans' Administration Hospital, Washington, DC, U.S.A.). Cells were grown at 37°C as monolayer cultures on 100mm Falcon plastic Petri dishes in modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum and 5 μ g of gentamycin/ml, with humidified atmosphere of air/CO₂ (9:1) (Dawson, 1979). Cells were harvested mechanically with a rubber 'police-man' and washed twice with 0.15M-sucrose before being freeze-dried.

Irradiation procedure

Samples of cell suspensions in 0.15M-sucrose (0.8ml; 2–4mg of protein) were added to 2ml or 4ml thin-wall glass ampoules and freeze-dried. The ampoules were then flushed with N₂ gas and sealed with a gas/O₂ torch. Samples were irradiated with a beam of high-energy electrons derived from a 16MeV linear accelerator (Addenbrooke's Hospital, Cambridge, U.K.) at a dose rate of approx. 2.0Mrad/min at 0–10°C, under conditions designed to minimize inactivation due to secondary effects such as free-radical formation (Levinson & Ellory, 1974); the accelerator was calibrated by Perspex [poly(methyl methacrylate)] dosimetry. Irradiated samples were rehydrated in 1.5ml of 50mM-Tris/HCl buffer, pH7.4, and assayed after 30min at 25°C for lysosomal hydrolase activity (McLawhon *et al.*, 1983a).

Assays of lysosomal hydrolase activities

Most lysosomal hydrolase assays were carried out on sonicated cell extracts in 0.1M-sodium citrate/sodium phosphate buffer, pH4.4, with 4-methylumbelliferyl glycoside substrates as described previously (Dawson & Tsay, 1977; Chien & Dawson, 1980). The optimum pH used for α -fucosidase assay was 4.9. Sphingomyelinase activity was determined with ceramido-phospho-[¹⁴C]choline (purchased from New England Nuclear) as substrate in 0.1M-citrate/phosphate buffer, pH4.4, containing 0.4mg of Triton X-100/ml. Liberated [¹⁴C]choline was separated in the aqueous (upper) phase of a chloroform/methanol/water (10:5:3, by vol.) partition as described previously (Dawson & Dawson, 1982) and radioactivity was determined by liquid-scintillation counting. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Data presented are means $\pm$ S.E.M. for triplicate determinations.

Determination of molecular sizes

Estimates of the molecular size of biological molecules were obtained by the application of classical target-volume theory to radiation inactivation data (Kepner & Macey, 1968). A relationship between the molecular mass and the dose of radiation required to decrease lysosomal hydrolase activity to 37% of normal (D_{37}) in megarads enabled molecular size to be calculated as $M_r = 6.4 \times 10^6/D_{37}$. The D_{37} value was obtained by linear regression analysis of the data [Plotrax 2 software by Omicron Engineering Science (Atlanta, GA, U.S.A.) with an IBM-PC computer].

Results

Freeze-drying of cell preparations in 0.15M-sucrose and subsequent storage of freeze-dried material in sealed glass ampoules for up to 90 days at room temperature had no adverse effects on either specific activity of untreated samples or the pH optimum of the residual lysosomal hydrolase activity. As an example, determination of the pH optimum of *N*-acetyl- β -glucosaminidase activity showed a peak between 4.0 and 4.6 both before and after treatment with 5.5Mrad of radiation, even though irradiation decreased absolute activity to 39% of control values (Fig. 1). This pH optimum was also in good agreement with similar determinations carried out on freshly harvested cells in the absence of 0.15M-sucrose.

The results of a large number of electron inactivation studies with 12 different substrates for acidic (lysosomal) hydrolases are summarized in Table 1. Molecular masses calculated from D_{37}

Fig. 1. pH optimum of *N*-acetyl- β -hexosaminidase activity in untreated freeze-dried NCB-20 cells (○) compared with cells treated with 5.5 Mrad of radiation (●), which destroys approx. 60% of the enzyme molecules

Enzyme activity is expressed as nmol of 4-methylumbelliferol liberated from 4-methylumbelliferyl *N*-acetyl- β -glucosaminide/h per mg of protein.

Table 1. Apparent molecular size of lysosomal hydrolases *in situ* as determined by electron inactivation analysis, compared with literature values

All values are the means for at least three separate determinations, D_{37} values being obtained by linear regression analysis of the data. Data for β -galactosidase were extrapolated to obtain the D_{37} value, since 10.5 Mrad was slightly above the practical reliable range (10 Mrad) of our procedures. [RI] indicates a molecular size determined by radiation inactivation methods. Values in parentheses indicated reported subunit sizes.

Enzyme	Target size by radiation inactivation (kDa)	Reported molecular size (kDa)	Reference
<i>N</i> -Acetyl- β -glucosaminidase	110 ± 5	100	Muhuren & Lowden (1980)
<i>N</i> -Acetyl- β -galactosaminidase	108 ± 4	107	Geyer & Arnon (1976)
β -Galactosidase	62 ± 3	70	Norden <i>et al.</i> (1974)
		64	Skudlarek & Swant (1979)
		64	Hoogetveen <i>et al.</i> (1984)
α -Galactosidase	112 ± 6	105	Dean & Sweeley (1979)
Arylsulphatase A	110 ± 5	105	Draper <i>et al.</i> (1971)
β -Glucuronidase	195 ± 15 (mouse)	390 (77, 60, 18)	Brot <i>et al.</i> (1978)
	65 ± 5 (human)	280	Tulsiani <i>et al.</i> (1975)
		85 [RI]	Nguyen Hong <i>et al.</i> (1980)
α -Fucosidase	155 ± 11	200 (50)	Alhadeff <i>et al.</i> (1975)
		150	Alhadeff & O'Brien (1977)
		150	Chien & Dawson (1980)
Sphingomyelinase	105 ± 15	130	Yamanaka & Suzuki (1982)
α -Glucosidase	150 ± 17	70	Steckel <i>et al.</i> (1982)
β -Glucosidase	120 ± 11	56	Furbish <i>et al.</i> (1977)
		67 [RI]	Potier <i>et al.</i> (1983)
β -Mannosidase	125 ± 15	Not reported	

values (as indicated in the Materials and methods section) are compared with representative values from the literature, which were obtained by a wide variety of physicochemical methods.

With both 4-methylumbelliferyl *N*-acetyl- β -galactosaminide and 4-methylumbelliferyl *N*-acetyl- β -glucosaminide as substrates, human skin fibroblasts cells gave comparable D_{37} values of 6.0 (Fig. 2a) and 5.97, corresponding to molecular masses of 107 ± 4 kDa and 108 ± 5 kDa respectively. The logarithmic decay curves were simple, with no evidence of biphasic regions, and the values obtained are very close to the accepted sizes of both *N*-acetyl- β -hexosaminidase A and *N*-acetyl- β -hexosaminidase B. When rodent NCB-20 hybrid cells were used for the study, values of 110 kDa (for 4-methylumbelliferyl *N*-acetyl- β -galactosaminide) and 112 kDa (for 4-methylumbelliferyl *N*-acetyl- β -glucosaminide) were obtained; these were not significantly different from those obtained for human tissue.

Similar good agreement with published values was also obtained for β -galactosidase (62 kDa), α -mannosidase (110 kDa), α -fucosidase (155 kDa), arylsulphatase A (110 kDa), sphingomyelinase (105 kDa) and α -galactosidase (105 kDa) (Table 1 and Figs. 3a and 3b). With the exception of arylsul-

phatase A, where the rodent tissue target size was half (55 kDa) that of the human enzyme, values from human and rodent material were indistinguishable. This is clearly seen for α -galactosidase, which gave D_{37} values of 5.7 for human skin fibroblasts and 5.8 for the mouse $\times$ Chinese-hamster hybrid NCB-20 cells (Figs. 3a and 3b).

Other lysosomal hydrolases gave target sizes that did not agree with published values, although some corresponded to hypothetical dimers or trimers. Good examples were β -glucosidase (120 kDa compared with 56 kDa) and α -glucosidase (150 kDa compared with 70 kDa). β -Glucuronidase in rodent tissue gave consistently high values (195 ± 15 kDa) (Fig. 4a), but not as high as the generally observed size of 390 kDa (with subunits of 77 or 60 kDa) or 280 kDa. However, more recent studies on human skin fibroblasts suggested a value of 65 kDa, which is close to the generally accepted subunit size for the human placental enzyme.

The advantage of the technique whereby reliable values for molecular mass can be obtained from whole-tissue samples is in the provision of further evidence in situations where the enzyme has not yet been purified. Thus the value of 125 kDa for β -mannosidase should be of use in future purification attempts.

Fig. 2. Comparison of target size: analysis of *N*-acetyl- β -galactosaminidase activity (a) in freeze-dried human skin fibroblasts and *N*-acetyl- β -glucosaminidase activity (b) in freeze-dried NCB-20 cells at -20°C

Radiation-inactivated cells were reconstituted in the appropriate buffer and assayed with 4-methylumbelliferyl substrates. Enzyme activities are expressed as nmol of 4-methylumbelliferol liberated/h per mg of protein.

Discussion

Because of their association with human inherited diseases, a large number of lysosomal hydrolases have been purified as the first step in enzyme replacement therapy, and many of their biochemical properties have been characterized. However, the molecular size reported for these enzymes varies widely and seems to be dependent on the methods of isolation, storage and determination of molecular mass. We therefore used a method for determining target size of the enzymes *in situ* (radiation inactivation) that has previously been successfully used for a large number of soluble and membrane-bound enzymes.

The molecular masses of the enzymes *N*-acetyl- β -hexosaminidase A and *N*-acetyl- β -hexosaminidase B, which hydrolyse both 4-methylumbelliferyl *N*-acetyl- β -galactosaminide and 4-methylumbelliferyl *N*-acetyl- β -glucosaminide equally well, is generally accepted as being in the region of 100–107 kDa for human tissue such as spleen (Muhuren & Lowden, 1980) and placenta (Geyer & Arnon, 1976). Since *N*-acetyl- β -hexosaminidase A consists of two 50 kDa dimers ($\alpha\beta$)

Fig. 3. Target-size analysis of α -galactosidase in freeze-dried human skin fibroblasts (a) and NCB-20 cells (b) at -20°C

Radiation-inactivated cells were reconstituted in the appropriate buffer and assayed with 4-methylumbelliferyl substrates. Enzyme activities are expressed as nmol of 4-methylumbelliferol liberated/h per mg of protein.

and *N*-acetyl- β -hexosaminidase B of two identical 50 kDa dimers (β_2), our value of 108–110 kDa corresponds to the dimer and is in good agreement with the generally accepted size of the functional form of these enzymes. A further complication is that many of the sphingolipid hydrolases such as *N*-acetyl- β -hexosaminidase A are also associated with activator proteins with a molecular size in the region of 20–30 kDa (Li & Li, 1976). Although this additional peptide is not covalently linked to the catalytic peptide, and would not therefore be expected to participate in energy transfer (Kempner & Miller, 1983), it may be reflected in the aggregate target size if it is indeed required for hydrolysis. An analogous peptide activator has also been implicated in the action of other sphingolipid hydrolases such as sphingomyelinase, α -galactosidase, β -glucosidase and arylsulphatase A. Sphingomyelinase was assayed with the natural substrate and gave a target size of 105 kDa, compared with literature values of 65 kDa (Yamanaka & Suzuki, 1982). More recently, values of 86 kDa or 94 kDa (depending on assay conditions) have been reported for sphingomyelinase by radiation inactivation techniques (a ^{60}Co -irradiator) (Maret *et al.*, 1984), which tends to confirm

Fig. 4. Comparison of target-size analysis (by radiation inactivation) of β -mannosidase (a) and β -glucuronidase (b) in the same sample of freeze-dried NCB-20 cells at 20°C

Under these conditions the δ -opiate (enkephalin) receptor gave a target size of 205 kDa. Enzyme activities were determined with the appropriate 4-methylumbelliferyl substrate under previously determined optimum conditions, and are expressed as nmol of 4-methylumbelliferol liberated/h per mg of protein.

that other peptides may indeed participate in the target size when natural substrates and detergents are employed. However, when synthetic substrates are used the results are much easier to interpret.

Thus the target size of β -galactosidase, when assayed with 4-methylumbelliferyl β -galactoside, gave a value of 62 kDa, confirming an earlier value that is close to the 70 kDa reported for the human liver enzyme (Norden *et al.*, 1974) and the 64 kDa reported for the mouse macrophage enzyme (Skudlarek & Swant, 1979). Hoogeveen *et al.* (1984) have reported that in human skin fibroblasts a 64 kDa monomer aggregates into a high-molecular-mass (600–700 kDa) complex together with a membrane-associated neuraminidase and a small (32 kDa) 'protective' protein, and that this is the normal functional size of the enzyme. Our 62 kDa value suggests that, when monomers of non-covalently linked complexes are themselves enzymically active, this low molecular size is the one obtained by radiation inactivation analysis. When the monomers are not enzymically active, as with glutamate dehydrogenase, Kempner & Miller (1983) have shown that radiation inactivation

analysis does not give the monomeric size. Neuraminidase is a relatively unstable enzyme and difficult to measure reliably in other than very fresh tissue. Nevertheless, γ -radiation inactivation has been successfully used to obtain values of 56 kDa for rat liver (Beauregard & Potier, 1982) and 240 kDa (cytosolic) and 203 kDa (lysosomal) for human leucocyte activity that hydrolyses 4-methylumbelliferyl *N*-acetylneuraminate (Nguyen Hong *et al.*, 1980). At present, it is not clear whether or not this enzyme is part of the large β -galactosidase complex.

With the use of this technique, Nguyen Hong *et al.* (1980) also reported a value of 85 kDa for human β -glucuronidase, which approximates to the 77 kDa and 60 kDa subunit sizes generally accepted for this enzyme (Brot *et al.*, 1978). However, a much larger molecular size is normally obtained for β -glucuronidase by conventional methods, namely 280 kDa or larger by gel filtration of the rat preputial-gland enzyme (Tulsiani *et al.*, 1975) and 380 kDa for human tissue (Brot *et al.*, 1978). Our value of 195 kDa for rodent cells is more in agreement with these larger values and was very consistently obtained.

Our studies thus far have shown very little difference in the target size of human or rodent forms of the enzymes studied, with the exception of β -glucuronidase and arylsulphatase A. Others have reported major size differences between human leucocyte (>200 kDa) and rat liver (56 kDa) forms of lysosomal neuraminidases (Nguyen Hong *et al.*, 1980; Beauregard & Potier, 1982) with the use of this technique. One of the major advantages of the radiation inactivation technique is that molecular masses can be obtained on whole-cell preparations without any need for enzyme purification. From studies of this type, we predict a functional target size of 125 kDa for β -mannosidase (Fig. 4a), an enzyme that has not yet been purified from mammalian tissue. By analogy with other related enzymes, this probably indicates a monomeric size of 60–70 kDa, information that should be of value in future purification attempts.

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