Effects of pH, detergent and salt on aggregation of Chinese-hamster-ovary-cell lysosomal enzymes

Michael J. BUCKMASTER, Andrea L. FERRIS and Brian STORRIE*

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

Upon detergent or hypo-osmotic lysis of CHO-cell postnuclear supernatants or isolated lysosomes at pH 4.8, the lysosomal enzymes β -hexosaminidase, β -galactosidase, α -fucosidase and cathepsin C were readily pelleted, whereas the exogenous marker, long-term-internalized horseradish peroxidase, was not. Salt or pH elevation greatly decreased lysosomal-enzyme pelletability. The results suggest that, under native conditions, lysosomal hydrolases may be aggregated. Aggregation could promote enzyme retention within the organelle.

INTRODUCTION

Speculations that lysosomal components aggregate to generate a lysosomal matrix and to control lysosomal enzyme activities are almost as old as the lysosome concept itself (for a review of earlier literature, see Koenig, 1969). General acceptance of these speculations has been limited because of long-standing observations (e.g., see Claude, 1946) that many components of crude subcellular preparations spontaneously aggregate at acid pH values typical of the lysosomal interior. Much of the evidence for associations of lysosomal components could be due to non-specific aggregation.

The best established case of physiological complexing of lysosomal components comes from genetic and biochemical studies of galactosialidosis, an autosomal recessive inherited lysosomal storage disease in humans (Wenger *et al.*, 1978; Hoogeveen *et al.*, 1980). Fibroblasts from patients are deficient in both β -galactosidase and sialidase on account of the absence of 32 kDa protein that complexes with the two enzymes to protect them from proteolytic digestion (d'Azzo *et al.*, 1982; Hoogeveen *et al.*, 1983).

In the present work we have investigated whether complexing of lysosomal enzymes may be a more general phenomenon. Postnuclear supernatants or highly purified lysosomes from CHO cells, a cell line of fibroblastic origin, were lysed by either hypo-osmotic shock or detergent treatment. We found that, under low-salt acid-pH conditions, native lysosomal enzymes pelleted, whereas the exogenous lysosomal marker, internalized horseradish peroxidase (HRP), did not. These results suggest that lysosomal hydrolases may be associated in a native matrix *in situ*.

MATERIALS AND METHODS

Cell culture

CHO cells were grown in suspension culture (Pool *et al.*, 1983) in Eagle's minimal essential medium (MEM), α -modification (α -MEM; Stanners *et al.*, 1971) without ribonucleosides or deoxyribonucleosides and supplemented with 10% (v/v) fetal-bovine serum (FC10).

HRP uptake conditions

Exponential-phase 220 ml suspension cultures (2×10^5 cells/ml initially) were incubated with HRP (0.25 mg/ml; type II; Sigma Chemical Co., St. Louis, MO, U.S.A.) for 16 h at 37 °C in α -MEM/FC10. These uptake conditions have been previously shown to localize HRP activity to lysosomes (Storrie *et al.*, 1984). Cells were harvested by centrifugation at 25 °C and washed repeatedly in warm α -MEM/FC10 to remove exogenous HRP.

Enzyme assays

HRP (EC 1.11.1.7) was assayed spectrophotometrically with *o*-dianisidine as substrate (Pool *et al.*, 1983). β -Galactosidase (EC 3.2.1.23), β -hexosaminidase (EC 3.2.1.52) and α -fucosidase (EC 3.2.1.51) activities were routinely assayed by using methylumbelliferyl substrates in the presence of 0.1% Triton X-100 (Pool *et al.*, 1983). Cathepsin C (EC 3.4.14.1) activity was assayed using glycyl-L-phenylalanine β -naphthylamide as substrate (Peters *et al.*, 1972). The latency of enzyme activity was determined as described by Pool *et al.* (1983) and Casey *et al.* (1986). Latent enzyme activity was defined as activity assayed in the presence of detergent minus that assayed in the absence of detergent.

Cell fractionation

Cells were disrupted by nitrogen cavitation and further homogenized by four strokes with a Potter–Elvehjem homogenizer (Pool *et al.*, 1983). Cell breakage was approx. 70%. A postnuclear supernatant (PNS) was prepared by centrifugation (Pool *et al.*, 1983). Lysosomes were isolated by centrifugation of the PNS first in a 'hybrid' Percoll/Metrizamide step gradient followed by flotation of the lysosomal fraction in a step Metrizamide gradient to separate lysosomes from mitochondria (Madden *et al.*, 1987).

Organelle lysis

For hypo-osmotic shock, postnuclear fractions or isolated lysosomes in 0.25 M-sucrose were diluted to a sucrose concentration of 0.04 M with 5 mM-sodium

Abbreviations used: CHO, Chinese hamster ovary; HRP, horseradish peroxidase; α -MEM, α -modification of Eagle's minimal essential medium; FC10, 10% (v/v) fetal-bovine serum; PNS, postnuclear supernatant.

* To whom correspondence and reprint requests should be addressed.

Fig. 1. Effect of lysis pH on pelletability of β -hexosaminidase, β -galactosidase and long-term-internalized HRP

CHO cells were incubated with HRP (0.25 mg/ml) for 14 h. At the end of this uptake, HRP had accumulated in lysosomes (Storrie *et al.*, 1984). Cells were then harvested and a total postnuclear fraction prepared. The PNS was divided into portions and the organelles lysed by hypo-osmotic shock at 4 °C. A pellet fraction was prepared by centrifugation at 100 000 g_{av} for 1 h. Enzyme activities were assayed in the presence of Triton X-100. Total recovery of enzyme activities between pellet and supernatant was > 90%. ●, β -Galactosidase (GALase); ■, β -hexosaminidase (HEXase); ▲, HRP.

acetate buffer, pH 4.8 or 5.4, or 5 mM-sodium phosphate or potassium phosphate buffer, pH 6.2 or 7.0, at 4 °C in most experiments. The diluted fractions were incubated for 15 min at 4 °C and then centrifuged at 100 000 g_{av} for 1 h in a Beckman Ti50 rotor at 4 °C to separate membranes and high- M_r aggregates from the soluble or supernatant fraction. Pellets were resuspended in 0.05% Triton X-100. Control experiments with overlapping-pH acetate and phosphate buffers gave similar results.

For detergent lysis, postnuclear fractions or isolated lysosomes in 0.25 M-sucrose were combined with 2 vol. of detergent solution in 5 mM-sodium acetate buffer, pH 4.8, or sodium phosphate buffer, pH 7.0. In some experiments, 0.25 M-sucrose, 100 mM-NaCl or bivalent cations were included in the detergent solution.

RESULTS AND DISCUSSION

The normal pH of the lysosome is about 4.6–4.8 (Ohkuma & Poole, 1978). Any physiological association of lysosomal enzymes ought to be most structured at an acidic pH (Hoogeveen *et al.*, 1983). In initial tests for such association, postnuclear supernatants were prepared from CHO cells that had accumulated the fluid-phase pinocytic marker, HRP, in a lysosomal compartment. Portions of PNS were then hypo-osmotically shocked over the pH range 4.8–7.0, and recovery of lysosomal enzyme activities in the 100 000 g_{av} pellet and the supernatant quantified. The distribution of the exogenous lysosome-resident enzyme, HRP, was assayed as an indicator for possible non-specific aggregation or trapping of lysosomally resident proteins. Before hypo-osmotic shock, the activities of β -galactosidase and β -hexosaminidase, native lysosomal enzymes, and of long-term-internalized HRP, were > 90% latent. After hypo-osmotic shock, irrespective of pH, all activities were non-

Fig. 2. Effect of detergents, pH and salt on the pelletability of β -galactosidase (□) and β -hexosaminidase (▨)

CHO cells were harvested and a total PNS prepared. The PNS was divided into portions. These portions were either diluted with 0.25 M-sucrose (no lysis) or with detergent solutions at 4 °C. Pellet fractions were prepared by centrifugation at 100 000 g_{av} for 1 h. Total recovery of enzyme activities between pellet and supernatant fraction was more than 90%. Abbreviations: GALase, β -galactosidase; HEXase, β -hexosaminidase; TX, 0.2% Triton X-100; Zw, 0.2% Zwittergent 3-12; Bj, 0.2% Brij 58; Suc, 0.25 M-sucrose; Na, 0.1 M-NaCl.

Fig. 3. Effect of detergent, salt and pH on the pelletability of four native lysosomal enzymes from isolated lysosomes

Lysosomes were isolated from a total PNS as described by Madden *et al.* (1987). Lysosomal samples were diluted 1:2 with either 0.25 M-sucrose (control, □), 0.2% Triton X-100 in acetate buffer, pH 4.8 (▨), 0.2% Triton X-100 (pH 4.8)/0.1 M-NaCl (■) or 0.2% Triton X-100 in phosphate buffer, pH 7.0 (▩) at 4 °C. Pellet fractions were prepared by centrifugation at 100 000 g_{av} for 1 h. Abbreviations: HEXase, β -hexosaminidase; GALase, β -galactosidase; FUCase, α -fucosidase; CATH C, cathepsin C.

latent. As shown in Fig. 1, β -galactosidase and β -hexosaminidase were highly pelletable after hypo-osmotic shock at acid pH, but not at neutral pH. In striking contrast, the pelletability of HRP was low over the entire pH range 4.8–7.0. These data are suggestive that any complexing of lysosomal components *in situ* is specific for native lysosomal proteins.

To test whether membrane association or other weak interactions are responsible for the high pelletability of lysosomal enzymes at acid pH, the effect of mild detergent and salt on β -galactosidase and β -hexosaminidase pelletability was determined. Detergent should solubilize the lysosomal membrane, and salt should disrupt weak interactions mediated, for example, by ionic bonds. As shown in Fig. 2, incubation of the PNS with the non-ionic detergents, Triton X-100 or Brij 58 at 0.2%, in the presence of 5 mM-sodium acetate buffer, pH 4.8, resulted in a small decrease, about 10–40%, in the pelletability of the two reference lysosomal enzymes, β -galactosidase and β -hexosaminidase. Incubation of the PNS with 0.2% of the zwitterionic detergent Zwittergent 3–12 at pH 4.8 caused about a 50% decrease in reference-enzyme pelletability. No further effect on reference-enzyme pelletability was observed as the conditions of any of the three detergents was increased to 1%. Under iso-osmotic conditions (0.25 M-sucrose), all detergent concentrations between 0.2% and 1% rendered the lysosomal enzymes non-latent. The addition of 100 mM-NaCl in the presence of detergent resulted in a further 50% decrease in reference-enzyme pelletability.

We conclude from these results that complexing/aggregation of lysosomal enzymes in a PNS lysate is due to weak interactions in which membranes do not play a critical role. Hohman & Bowers (1984) have previously suggested that the pelletability of lysosomal enzymes in *Acanthamoeba* lysates is due to enzyme association with the lysosomal membrane. In their work, the effect of detergents was not investigated.

To test whether the pelletability of lysosomal enzymes in PNS lysates is due to interactions with non-lysosomal components, highly purified CHO-cell lysosomes (Madden *et al.*, 1987) were used as starting material. As shown in Fig. 3, for control isolated lysosomes, the four lysosomal enzymes tested were greater than 70% pelletable at 100 000 g_{av} . After treatment of the lysosomes with 0.2% Triton X-100 at pH 4.8, the enzymes were no longer latent, and their pelletability was high. The reference lysosomal enzymes, β -galactosidase and β -hexosaminidase, appeared to fall into one group, at 80–90% of control. α -Fucosidase and cathepsin C, on the other hand, at almost 50% of control, fell into a second group. As expected, the addition of NaCl or elevation of the lysis pH to neutrality decreased the pelletability of all enzymes. It should be noted that the decrease in pelletability was decidedly greater than that observed for the PNS. This may relate to the presence of 'sticky' complexes such as chromatin released by cell homogenization in the PNS. No stabilization of lysosomal enzyme aggregation against salt or pH elevation was observed with the addition of 1 mM- Ca^{2+} and 0.5 mM- Mg^{2+} . Similar results were seen with 1% Triton X-100. These detergent results suggest that the observed aggregation of lysosomal hydrolases does not require continuous association of the enzymes with the lysosomal membrane.

The detergent-stable aggregation of four native lysosomal hydrolases under low-salt acid-pH conditions

is consistent with the complexing of lysosomal components *in situ* to generate a lysosomal matrix. *In vivo* such aggregation of lysosomal enzymes could be important in preventing reciprocal exchanges between lysosomes and endosomes. Such reciprocal exchanges would lead to the loss of lysosomal enzymes from the organelles. For fibroblasts, little if any exocytosis of native acid hydrolases occurs from the lysosome (see, e.g., Gonzalez-Noriega *et al.*, 1980). Likewise for fibroblasts, little if any exocytosis occurs for macromolecules internalized into a lysosomal compartment (Steinman *et al.*, 1974; Storrie *et al.*, 1984; Buckmaster *et al.*, 1987). Conceivably, a lysosomal matrix may also trap exogenous molecules. Preliminary investigations (M. J. Buckmaster & B. Storrie, unpublished work), using osmotic detergent lysis conditions to solubilize the lysosomal membrane, have shown that, for PNS preparations, iso-osmotic lysis with 0.2% Brij 58 results in the co-pelleting of long-term-internalized HRP with β -galactosidase and β -hexosaminidase.

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